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# Somaclonal Variation in Hibiscus Acetosella Welw. Ex Hiern: Altered Fertility and Floral Ontogeny.

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# SOMACLONAL VARIATION IN *HIBISCUS ACETOSELLA* WELW. EX HIERN: ALTERED FERTILITY AND FLORAL ONTOGENY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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in

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by
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ABSTRACT

A study was undertaken to examine fecundity and floral ontogeny in wild type (WT) and tissue culture-derived (TC) plants of Hibiscus acetosella Welw. ex Hiern. Twenty-eight plants regenerated in vitro from somatic embryos were outplanted and established in the greenhouse, as were three WT plants from seed. The TC and WT plants were self-pollinated. Nine of the TC plants were self-fertile but exhibited a reduction in fruit set and seed set when compared to WT. Days to maturity for fertile fruit varied signicantly among TC plants. There was no correlation of days to maturity and number of seed set. Days to abortion of nonfertile fruit varied significantly among TC plants. Data suggested both pre- and post-fertilization, self-incompatibility barriers were present in the TC plants. Comparison of days to abortion for self-pollinated fruit and days to abortion for emasculated flowers indicated that pre-fertilization barriers to fertility occurred within eight to ten days of pollination.

The centripetally initiated floral organs in WT consisted of seven to ten bracteoles, five sepals with a congenitally connate basal tube and free terminal lobes, five petals, a connate androecial tube bearing numerous centrifugally initiated stamens in two rows opposite each petal, and five carpels with a connate ovary base, postgenitally fused compound style, and five stylodes. The

sepals were valvate in bud, the petals convolute. The number of floral organs in the TC plants varied significantly: fewer stamens than in WT; all other organs either higher or lower than WT. The TC plants had abnormalities in primordia size, relative position within and between whorls, sepal and petal aestivation, connation of the calyx and androecial tubes, and postgenital fusion of the styles. Centripetal organ initiation between whorls, centrifugal androecial primordia initiation, and connation of the gynoecial tube were not affected. Petalody of the androecium occasionally was observed. Ovule formation on the androecial tube was common and paralleled gynoecial ovule development. The androecial ovules contained functional-appearing embryo sacs.

# INTRODUCTION

The ability to culture plant organs, tissues, or even single cells *in vitro* on the appropriate nutrient medium has resulted in numerous fundamental investigations into plant physiology, genetics, and cytology. Tissue culture has also offered an alternative method of plant propagation, such that today entire ornamental industries are based on the ability to culture and proliferate plant materials *in vitro*.

Plant proliferation via tissue culture depends on several different growth processes *in vitro*. Shoot numbers can be increased through proliferation of axillary buds, via organogenesis, the formation of *de novo* shoots either directly from pre-existing plant tissues or from callus, or via somatic embryogenesis, the formation of embryos from either pre-existing plant tissues or from callus.

The process of somatic embryogenesis has attracted both fundamental and applied research. The formation of embryos *in vitro* can parallel zygotic embryogenesis; as such, this offers the opportunity to examine the physiology and cytology of embryogenesis in an isolated environment. Somatic embryogenesis has also generated attention as a propagative system, as it is theoretically possible to regenerate thousands of somatic embryos from a single explant in a relatively short time. This investigation is based on the ability to readily regenerate somatic embryos *in vitro* from one particular plant, *H. acetosella*.

In recent years it has become apparent that many plants regenerated *in vitro* are not true to type, or exhibit variation from the parental plant material. This manifestation, known as somaclonal variation, arises from the accumulation *in vitro* of genetic and epigenetic variations within the plant tissues. The research on these variations has been two-fold: first, to take advantage of the variations by screening for novel mutations that can be used in a plant improvement program, and second, to determine the genetic, physiological, or cytological mechanisms underlying these variations.

In order to introduce useful variations arising from tissue culture into a plant improvement program, it is necessary that these traits be heritable. However, somaclonal variation often depresses fertility of the tissue culture regenerated plants. Chapter One of this investigation presents the fecundity of tissue culture regenerated plants of *H. acetosella*.

Somaclonal variation also offers the opportunity to examine alterations in other plant growth processes, such as floral organogenesis. One of the advantages of such evaluations is that it is possible to compare the variant plants with the parental material. Chapter Two of this investigation presents the floral ontogeny of wild type *H. acetosella*, and Chapter Three presents the floral variations in the tissue culture derived plants.

CHAPTER ONE

# Altered Fertility in Tissue Culture-Derived Plants of *Hibiscus acetosella*


## ABSTRACT

The fertility of twenty-eight tissue culture-derived (TC) plants was compared to that of three wild type (WT) plants of *Hibiscus acetosella* Welw. ex Hiern. Only nine of the TC plants were self-fertile. Fruit and seed set were higher for the WT plants than for any of the TC plants. One of the two outcrosses of self-sterile TC plants with WT pollen was fertile. Days to fruit maturity after pollination were fewer for most of the TC plants than for the WT. There was no correlation of days to maturity and number of seed set per fruit in the TC plants. Days after pollination to fruit abortion in the TC plants exhibited a bi-modal distribution. The interval of one to nine days after pollination to fruit abortion conceivably represents the presence of pre-fertilization barriers to fertility, and the range of 11 to 45 days represents the presence of post-fertilization barriers. Emasculated WT

flowers all aborted within 1 to 8 days; whereas, the fruit that aborted between 11 to 45 days contained only immature or nonviable seed.

## INTRODUCTION

Genetic variability in TC plants has been widely reported (Reisch, 1983; Evans and Sharp, 1986). Larkin and Scowcroft (1981) coined the expression "somaclonal variation" to describe the variability expressed in plants regenerated from somatic tissues. Somaclonal variation has been attributed to changes in chromosomal number, chromosomal rearrangements, nuclear gene mutations, and cytoplasmic gene alterations (Evans, Sharp, and Medina-Filho, 1984). The accumulation of genetic variations has been correlated with explant genotype and source, duration of _in vitro_ culture, and culture conditions (Evans and Sharp, 1986).

A potentially attractive usage of somaclonal variation is for crop improvement. Disease-resistant clones have been obtained from tissue culture of sugarcane and potato (Larkin and Scowcroft, 1981) and several agronomically useful tomato mutants have been obtained from selfings of tissue culture plants (Evans, _et al_, 1984). The scented geranium _Pelargonium graveolens_ 'Velvet Rose' was probably the first commercially released TC cultivar (Skirvin and Janick, 1976).

The application of somaclonal variation to plant improvement is contingent upon transmission of stable, useful genetic alterations to the progeny. Unfortunately, plants regenerated from tissue culture frequently exhibit a reduction or loss of fertility. While this may not be crucial in asexually propagated crops such as potato or sugarcane, it constitutes a barrier for the introduction of useful mutations into seed-propagated crops. Reduced fertility is often inferred to be due to aneuploidy (Evans and Sharp, 1986), though reduced fertility without a concomitant ploidy alteration has also been reported, such as in alfalfa (Pfeiffer and Bingham, 1984).

Limited research has been reported on the actual mechanisms within tissue culture regenerated plants which contribute to their reduced fertility. Syono and Furuya (1972) associated sterility in tobacco with abnormal pollen development. Hughes (1986) reported genetically based crossing barriers in tissue culture tobacco plants. In this report, we will describe reduced fertility in tissue culture-derived plants of Hibiscus acetosella and present evidence that suggests both pre-fertilization and post-fertilization fertility barriers.

## MATERIALS AND METHODS

Tissue culture and plant establishment. Hypocotyl, cotyledon, and root explants from seedlings of H. acetosella were placed in culture on media designated SEM-1 and RM-1.

Experimental conditions and media components have been previously described (Reynolds and Blackmon, 1983). Explants readily callused on both media in four to eight weeks. After callus formation, alternating sequential subcultures were made at six to twelve week intervals to induce somatic embryogenesis and increase callus proliferation. After five to seven subcultures, somatic embryos from SEM-1 were placed for germination on a medium designated AML-7, which consisted of full strength Nitsch and Nitsch's Medium H (Nitsch and Nitsch, 1969) minus 3-indoleacetic acid (IAA), full strength Gamborg's Medium $B_5$ (Gamborg, Miller, and Ojima, 1968) minus 2,4-dichlorophenoxyacetic acid (2,4-D), 10 g/liter sucrose, and 8.0 g/liter agar. This medium was demonstrated to be optimal for embryo germination and plantlet growth (Ault, Reynolds, and Blackmon, 1985). Clusters of approximately four to twenty somatic embryos were placed on 10 ml AML-7 in 20 X 150 mm glass culture tubes. After four to eight weeks clusters of 1 to 2 cm tall shoots with several small leaves and little or no root development were obtained. The shoots were then separated and placed for a second culture on 90 ml AML-7 in Magenta GA-7 Vessels. After six to ten weeks plants 5 to 10 cm tall with numerous leaves and good root systems resulted. Plants were removed from tissue culture between November 15, 1984, and August 4, 1985, potted in a mixture of 2 Pro Mix : 2 perlite : 1 peat moss (by volume), and placed under a clear polyethylene film in the

greenhouse. After two to three weeks, the polyethylene film was removed and surviving plants were either maintained in pots in the greenhouse or transplanted to the field.

Cuttings from the plants in the field were subsequently rooted under the same conditions used for establishment of the tissue culture plants. A total of 35 plants, some of which were from cuttings of the tissue culture plants and some the original tissue culture plants, were established in the greenhouse for this study. Three wild type plants grown from the seed lot used to establish the tissue culture cell lines were also planted in the greenhouse as controls.

Pollinations. The genotype of H. acetosella used in this study is a short-day plant with approximately a 12 hr dark requirement (personal observation). The TC and WT plants were in bloom from approximately November 1, 1985, until May 1, 1986, and pollinations were performed between November 4, 1985, and April 24, 1986. The flowers of H. acetosella typically open in the evening or morning and close by early afternoon. Flowers were tagged and pollinated between 8:00 am and 12:00 noon. Dehisced anthers were gathered with forceps and rubbed over the stigmatic surfaces of the same flower, which ensured pollination. The pollen of this species is "sticky", which helps preclude either pollen falling off the stigmas once pollinated or accidental wind-borne outcrossing with other flowers. Most flowers were self-pollinated; however, a limited number of

pollinations were conducted using WT pollen to pollinate two of the TC plants. Fruit development was monitored daily. Aborted flowers or fruit would fall from the plants or could be dislodged by a light tap to the receptacle. Fertile fruit (e.g. fruit containing seed) would turn brown and split along vertical lines of dehiscence at maturity but would persist on the plant until mechanically removed. Days to abortion or maturity and the number of fully developed seed (indicated by fullness of seed and brown seed coat) per fruit were tabulated. No fully developed seed were found in aborted fruit.

Fertile fruit were harvested after maturity and stored at room temperature. Although the capsules of H. acetosella dehisce on the plant, seed will not fall out of the dried fruit unless mechanically removed. Seed viability was assessed by nicking seed coats with a razor blade and planting in the greenhouse in flats containing 1 Pro Mix : 1 perlite (by volume). Emergence counts were tabulated eight weeks after sowing.

Emasculations. Flowers from three WT plants, five TC plants, and one seed-grown progeny of a TC plant were emasculated to tabulate days to abortion of nonpollinated flowers. The day before anthesis, which can be predicted by the exsertion of the corolla from the enveloping calyx, flowers were mechanically opened and the nondehisced anthers removed with forceps. Flowers were tagged then collected

daily as they aborted. Ten flowers were emasculated on each plant examined.

Chromosomal analysis. Root tips from greenhouse-grown plants were placed in 0.002 M solution of 8-hydroxyquinoline for four to six hours at 20°C for chromosome contraction. After the chromosomal contraction treatment, root tips were hydrolyzed in 5N HCl for five to ten minutes, dissected, squashed, and stained on microscope slides in an acetic acid-orcein stain solution (2.2 g orcein dissolved in glacial acetic acid, diluted to 45% acetic acid with distilled water for staining). Cell spreads were examined with a phase contrast Zeiss microscope at a magnification of 630X.

Statistical analysis. One-way analyses of variance (ANOVAs) were computed for mean seed production and mean days to dehiscence for pollinated flowers, and means days to abortion for pollinated and emasculated flowers. Mean values are reported plus or minus standard errors (SE). Dunnet's Test for comparison of treatment means with a control was used to compare mean seed production in the WT and TC plants. Nonsignificance in any test was at a .05 level of probability. Statistical analyses were computed on an IBM PC-XT using Microstat statistics software (Ecosoft, Inc.).

## RESULTS

The number of self-pollinated flowers for TC, WT, and selected crosses (TC X WT) are listed in Table 1. Flowers available for pollination varied as a result of uneven blooming among plants. Plants TC 1, TC 14, and TC 15 died prior to flowering. Plants TC 32, TC 33, TC 34, and TC 35 formed flower buds, but the buds on these plants aborted prior to anthesis. Plant TC 13 could not be self-pollinated as pollen did not form in the anthers.

Fruit set (e.g. fruit that persisted on the plant to maturity and contained seed that appeared to be fully developed) was high in the WT plants, averaging 94.9%; whereas, the maximum fertile fruit-set by the TC plants was 76.3% for TC 27. Fruit set in the remaining TC plants was generally poor, as 19 out of 27 plants did not carry any fruit to maturity (Table 1).

The two plants used in outcrossing, TC 11 and TC 13, were among those found to be self-infertile. Pollination of TC 11 with WT pollen restored fertility (Table 1). The attempted cross of TC 13 by WT pollen was barren, although days to abortion was the longest observed (Table 1).

The mean number of seed per fruit proved to be significantly different among the plants (Table 1, $F = 93.1$, $P < .001$). The WT plants set the greatest number of seed per fruit, averaging 16.8. The highest mean seed set for any TC plant was 4.8 for TC 8. There was a significant difference ($P = .05$) between WT and TC 8 seed production

according to Dunnet's Test (Dunnet value for a significant difference at P = .05 between WT and TC 8 is 2.22). The cross TC 11 by WT pollen produced only an average of 1.8 seed per fruit.

Meaningful comparisons of emergence percentages were difficult because seedlots were small for most of the fertile TC plants. Emergence for WT averaged 95.0%, while emergence for the two largest TC plant seedlots was 83.8% and 81.5% for TC 27 and TC 8, respectively. Although emergence of the other seed lots was in general high, the numbers of seed tested were variable and low (Table 1).

The average number of days after pollination to dehiscence for the fertile fruit varied significantly among the plants (Table 1, $F = 20.8$, $P < 0.001$). TC 4 had a higher mean than WT; whereas, all other fertile TC plants had lower means than WT plants. Fertile fruit of both WT and TC plants dehisced between 25 and 43 days after pollination. Correlation between number of seed produced per fruit and days to fruit dehiscence was marginally significant for WT ($r = .2362$, $t = 2.004$, $P < .05$; $r = .2356$ for nonsignificance) but was not significant for the pooled TC plants ($r = .0303$, $t = 0.29$, n.s.). This indicates that early maturation of the TC fruit (other than TC 4) in comparison with WT was not a result of lower seed set, as would be expected if days to fruit maturity was in part due to the nutritional supply of varying numbers of seed.

The average number of days after pollination to fruit abortion also varied significantly (Table 1, $F = 21.8$, $P < 0.001$) among plants. Plant TC 30 had the highest average, 24.0 days, and plant TC 25 had the lowest average, 2.9 days. The pooled data for TC plants exhibited a bi-modal response (Fig. 1D). One subpopulation ranging from one to ten days after pollination contained 59% and the second subpopulation ranging from eleven to forty-five days after pollination contained 41% of aborted fruit. Individually, the TC plants displayed one of three abortion patterns: all abortions falling within the first 10 days (Fig. 1A), all abortions after 10 days (Fig. 1B), or abortions divided into early and late groups (Fig. 1C). The most common pattern was the bi-modal distribution, which was observed in 19 out of 28 TC plants.

The two outcrosses had higher average days to fruit abortion than in any of the selfings, 30.1 days for TC 13 X WT and 26.1 days for TC 11 X WT. A comparison of the selfing and outcrossing data for TC 11 indicates a shift from early abortions when selfed to late abortions when outcrossed with WT pollen (Fig. 2).

When data for days to abort and days to dehisce are compared, 18% of the aborted fruit fall in the range observed for fruit dehiscence (Fig. 1D). Some of the late-aborting fruit dried, dehisced, and persisted on the plant as did fertile fruit but were scored as nonfertile due to lack of seed development. Instead, these fruit contained

what appeared to be unpollinated ovules and incompletely developed seed. The fertile fruit from TC plants invariably also contained numerous unpollinated ovules or immature seed, while the WT fruit contained few or no immature seed. Low seed set in the TC plants does not appear to be a function of decreased ovule production.

H. acetosella is a naturally occurring allotetraploid with 2n = 4x = 72 chromosomes (Menzel, Goetz, and Adamson, 1983). Small size, uneven spread, and large number of chromosomes made accurate chromosome counts difficult. However, approximate counts were made for plants TC 15, TC 16, TC 17, TC 19, and TC 24, all of which ranged from 54 to 60 chromosomes. It appears that the plants examined are all triploids or aneuploids approaching triploidy.

## DISCUSSION

The production of somaclonal variants from plant tissue cultures can be influenced by the duration of culture and culture conditions (Evans and Sharp, 1986). It has also been reported that long-term cultures tend to be genetically unstable (Reisch, 1983; Evans and Sharp, 1986). The TC plants employed in this study were regenerated from cell lines maintained as callus-embryogenic cultures for fifteen to eighteen months. The auxin 2,4-D used in both the embryogenic (SEM-1) and nonembryogenic media (RM-1) has been implicated as an inducer of genetic variation in numerous tissue culture systems (Bayliss, 1980). It has been

difficult to separate the effects of 2,4-D from duration of culture, as many of the reports on deleterious effects of 2,4-D also employed long-term cell cycles (Evans and Sharp, 1986). Both duration of culture and use of 2,4-D could have contributed to the observed variability in the TC plants.

Reduced fertility in tissue culture plants is frequently associated with alterations in chromosome number (Evans and Sharp, 1986). Only one of the plants examined for chromosome number was fertile, TC 17. This plant appears to be a triploid ($2n = 3x = 54$). Though triploid plants are generally sterile, the formation of nonreduced gametes can lead to partial restoration of fertility. For example, several clones of the triploid orchid hybrid Slc. Jewel Box are known to be fertile, though viable seed production is low.

Effects on the reproductive potential of all the TC plants were manifested at three levels; pollen development, fruit set and seed set. Seed viability was more difficult to interpret but may also have contributed to altered fertility.

Barriers known to inhibit self-compatibility in plants are generally categorized as pre-fertilization or post-fertilization. Pre-fertilization barriers, which prevent fertilization, include failure of pollen to develop, failure of pollen to germinate on the stigma, slow or incomplete growth of the pollen tube to the ovule, or inhibition of the pollen tube at or in the ovule (Hughes,

1986; Seavey and Bawa, 1986). Post-fertilization barriers, which retard or inhibit zygote and seed development, include embryo abortion, embryo-endosperm incompatibility, and seedling lethality (Brar and Khush, 1986). These incompatibility barriers, when visualized from a growth and development perspective, should segregate temporally after pollination and be reflected in fruit abortion patterns. For example, failure of pollen to germinate should result in fruit drop relatively soon after pollination; whereas, post-fertilization events such as embryo abortion may be expressed over a protracted interval.

Within this conceptual framework, TC plants with fruit abortion in less than ten days after pollination could be categorized as pre-fertilization, self-infertile. This contention is supported by comparison of fruit abortions from the selfings to abortion for emasculated flowers (Table 2). Though there was a significant difference among TC plants in mean days to abortion for emasculated flowers (Table 2, $F = 11.99$, $P < 0.001$), all of the abortions were within one to eight days. Evidence for post-fertilization barriers in the TC plants was the presence of immature seed in addition to what appeared to be nonfertilized ovules in aborted fruits, indicating fertilization most likely occurred, but was subsequently followed by eventual embryo or seed abortion for the fertilized ovules. Comparison of all the abortion data (Fig. 1D, Table 2) indicated pre-fertilization infertility in the TC plants occurred

within eight or nine days after pollination, and post-fertilization infertility occurred eleven days or later after pollination. As previously stated, post-fertilization infertilities lag temporally behind pre-fertilization infertility due to the longer duration necessary for fruit abortion caused by embryo abortion, endosperm incompatibility, and so forth. On this premise, TC plants can then be categorized to the type of self-infertility: TC 29 as pre-fertilization (Fig. 1A), TC 30 as post-fertilization (Fig. 1B), TC 8 as both pre- and post-fertilization (Fig. 1C), and so forth.

Both pre- and post-fertilization infertility barriers can occur in the same plant. Abortion of the TC 11 selfings occurred within six days of pollination, indicating infertility was pre-fertilization. The outcross of TC 11 by WT pollen yielded some viable seed, demonstrating that some of the TC 11 ovules were functional. However, both fruit set and seed set for the outcross of TC 11 X WT pollen were low, indicating that most of the TC 11 ovules were functionally incapable of development to maturity. The possibility of pre-fertilization infertility in the outcross was minimal, as fruit abortion all occurred eighteen days or more after pollination, indicating post-fertilization infertility was in effect. Syono and Furuya (1972) reported similar results working with tissue culture regenerated tobacco plants. Self-infertility was partially due to the production of nonviable pollen, as indicated by staining,

but aberrant ovule formation was also involved as no viable seed were obtained in crosses with wild type pollen.

The presumptive presence of both pre- and post-fertilization barriers would explain the low fruit and seed set observed in the TC plants. Fruit abortion will result from any pre-fertilization barriers to fertility. Post-fertilization barriers will decrease the number of ovules that develop to maturity; if none of the ovules are capable of development, then fruit abortion will result.

Chromosomal aberrations tend to accumulate over time in tissue culture systems (Evans and Sharp, 1986). It is not unreasonable to expect that similar alterations affecting fertility occurred in the H. acetosella TC plants. Specific genetic alterations may have arisen that resulted in the formation of pre- and post-fertilization barriers, as in the genetic crossing barriers derived from tissue culture regeneration of tobacco (Hughes, 1986). Alternatively, the loss of entire chromosomes, as in our sterile triploids or aneuploids, would remove some of the genetic regulatory machinery for normal reproductive processes. Fertility barriers that would not necessarily be analogous to traditional breeding barriers as specified by selective gene action would result. Without further outcrosses, reciprocal crosses, and cytological examinations of the TC plants, we cannot clearly specify what type(s) of breeding barriers were in effect.

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Table 1. Fertility of H. acetosella wild type (WT) and tissue culture-derived (TC) plants.

| Plant[a] | N[b] | Fruit set (%) | Seed[c] set | Emergence[d] (%) | Days to[e] dehiscence | Days[f] to abort |
|---|---|---|---|---|---|---|
| WT | 78 | 94.9 | 16.8 ± 0.5 | 95.0 (60) | 35.5 ± 0.3 | 6.0 ± 0.0 |
| TC 2 | 74 | 0.0 | - | - | - | 3.7 ± 0.4 |
| TC 3 | 73 | 0.0 | - | - | - | 14.8 ± 2.1 |
| TC 4 | 57 | 14.0 | 1.9 ± 0.4 | 53.3 (15) | 38.7 ± 1.0 | 7.2 ± 2.1 |
| TC 5 | 55 | 16.4 | 2.0 ± 0.4 | 40.9 (22) | 34.8 ± 1.5 | 9.2 ± 2.0 |
| TC 6 | 33 | 0.0 | - | - | - | 5.3 ± 1.3 |
| TC 7 | 58 | 0.0 | - | - | - | 6.8 ± 1.1 |
| TC 8 | 53 | 26.4 | 4.8 ± 0.9 | 81.5 (65) | 32.1 ± 0.7 | 10.1 ± 2.2 |
| TC 9 | 50 | 0.0 | - | - | - | 8.2 ± 1.4 |
| TC 10 | 104 | 1.0 | 1.0 ± 0.0 | 0.0 (1) | 32.0 ± 0.0 | 7.9 ± 1.3 |
| TC 11 | 99 | 0.0 | - | - | - | 3.1 ± 0.4 |
| TC 11 X WT | 20 | 25.0 | 1.8 ± 0.4 | 85.7 (7) | 33.8 ± 1.5 | 26.1 ± 1.3 |
| TC 12 | 60 | 0.0 | - | - | - | 7.3 ± 1.2 |
| TC 13[g] | - | - | - | - | - | - |
| TC 13 X WT | 49 | 0.0 | - | - | - | 30.1 ± 1.1 |
| TC 16 | 10 | 0.0 | - | - | - | 4.5 ± 1.5 |
| TC 17 | 78 | 14.1 | 1.1 ± 0.1 | 100.0 (11) | 30.3 ± 0.4 | 15.2 ± 1.8 |
| TC 18 | 17 | 5.9 | 1.0 ± 0.0 | 100.0 (1) | 33.0 ± 0.0 | 3.3 ± 0.3 |
| TC 19 | 89 | 0.0 | - | - | - | 13.1 ± 0.8 |
| TC 20 | 81 | 0.0 | - | - | - | 20.1 ± 1.5 |
| TC 21 | 57 | 7.0 | 1.3 ± 0.3 | 50.0 (2) | 30.1 ± 0.6 | 18.1 ± 1.3 |
| TC 22 | 13 | 0.0 | - | - | - | 18.0 ± 3.1 |
| TC 23 | 50 | 0.0 | - | - | - | 11.0 ± 4.9 |
| TC 24 | 61 | 0.0 | - | - | - | 23.3 ± 1.9 |
| TC 25 | 43 | 0.0 | - | - | - | 2.9 ± 0.3 |
| TC 26 | 38 | 0.0 | - | - | - | 11.4 ± 4.4 |
| TC 27 | 59 | 76.3 | 2.7 ± 0.3 | 83.8 (80) | 29.6 ± 0.4 | 19.3 ± 3.7 |
| TC 28 | 41 | 7.3 | 1.0 ± 0.0 | 33.3 (3) | 27.0 ± 0.0 | 17.5 ± 1.7 |
| TC 29 | 40 | 0.0 | - | - | - | 3.3 ± 0.2 |
| TC 30 | 42 | 0.0 | - | - | - | 24.0 ± 0.8 |
| TC 31 | 15 | 0.0 | - | - | - | 14.8 ± 0.6 |
| | | | F = 93.1[h] | | F = 20.8 | F = 21.8 |
| | | | P < 0.001 | | P < 0.001 | P < 0.001 |

[a] Plants self-pollinated except where indicated. Crosses were with pollen from WT. WT data are from three plants.
[b] Number of flowers pollinated.
[c] Mean seed set per fertile fruit ± SE.
[d] Seed emergence followed by number of seed tested in parentheses.
[e] Mean days to dehisce for fertile fruit ± SE.
[f] Mean days to abort for nonfertile fruit ± SE.
[g] Plant TC 13 not self-pollinated due to lack of pollen in anthers.
[h] F-statistics from one-way ANOVAs.

Table 2. Days to abort for emasculated flowers of *H. acetosella*.

| Plant[a] | Days to abort[b] |
|---|---|
| WT | 4.1 ± 0.3 |
| TC 4 | 2.5 ± 0.3 |
| TC 8 | 1.8 ± 0.2 |
| TC 8 R1 | 1.6 ± 0.2 |
| TC 15 | 4.6 ± 0.4 |
| TC 17 | 2.3 ± 0.2 |
| TC 27 | 2.8 ± 0.6 |
| Range | 1 - 8 |
| | F = 11.99[c] |
| | P < 0.001 |

[a] WT = wild type. TC 8 R1 = progeny of TC 8 by self. All other plants tissue culture-derived (TC).
[b] Mean days to abort ± SE based on 10 flowers per plant.
[c] F-statistics based on one-way ANOVA.

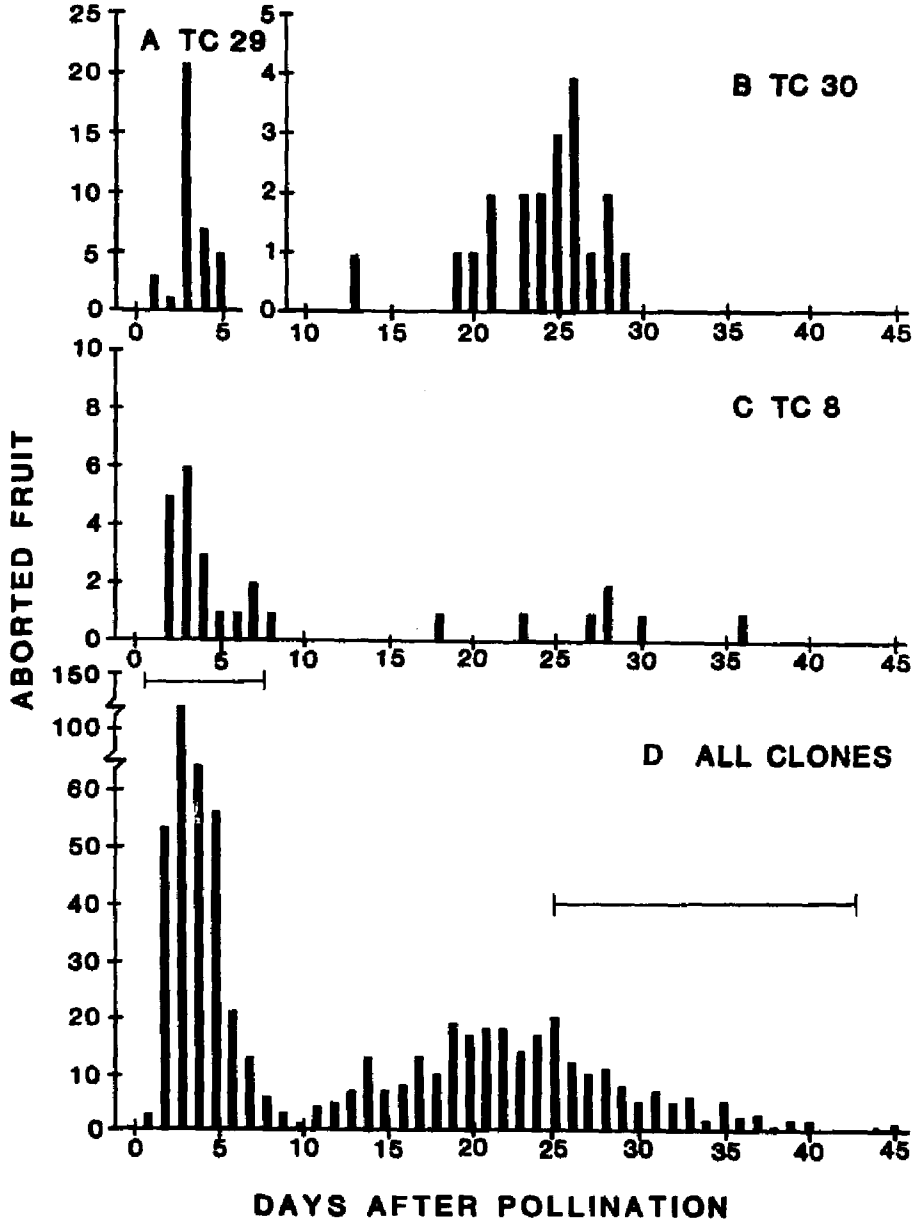


Figure 1. Aborted fruit of H. acetosella tissue culture-derived (TC) plants as a function of days after pollination. A. TC 29. B. TC 30. C. TC 8. D. All TC plants. The upper-left horizontal bar in D represents the range for days to abortion for all emasculated flowers, and the lower-right horizontal bar represents the range for days to dehiscence for all fertile wildtype and TC fruit.

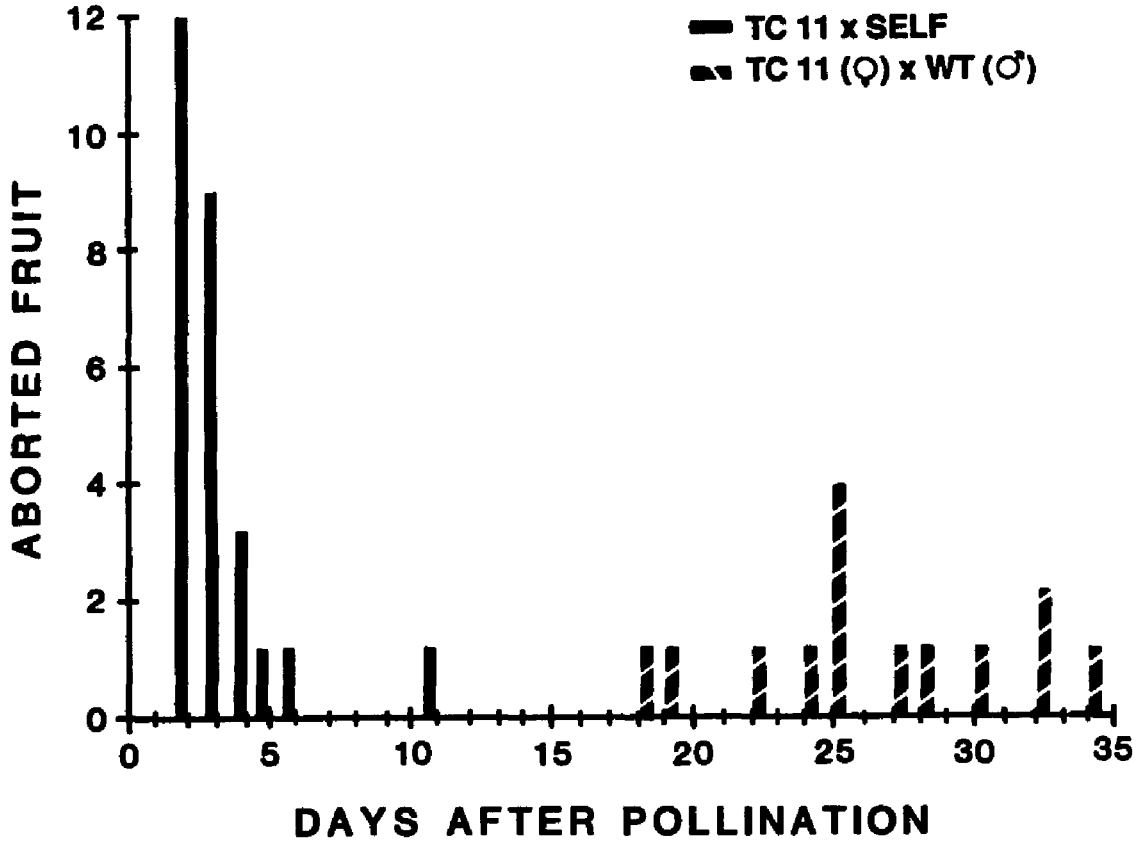


Figure 2. The number of aborted fruit as a function of days after pollination for the selfing of H. acetosella tissue culture-derived (TC) plant TC 11 and the outcross of TC 11 X WT.

## CHAPTER TWO

# Floral ontogeny in Hibiscus acetosella. I. Wild type


## ABSTRACT

Floral organs of Hibiscus acetosella Welw. ex Hiern are initiated in centripetal sequence. Seven to ten whorled bracteoles are initiated sequentially on the flank of the floral meristem. The five whorled sepal primordia are initiated more or less concurrently. The calyx forms five free distal lobes, their base a connate tube. The calyx lobes are valvate in bud. The five alternisepalous, whorled petals are initiated simultaneously or nearly so on the abaxial flank of the stamen ring primordium. The petals are obliquely inserted and are convolute in bud. The five antepetalous androecial primordia divide first laterally, then radially to form four whorls of primordia. These divide centrifugally to form radial pairs of primordia, which differentiate into bisporangiate stamens. Additional whorls of androecium primordia are initiated centrifugally, forming stamen pairs, single bisporangiate

stamens, or sterile filaments. The filament tube is connate. Each petal and two antepetalous stamen fascicles are supplied by a common trace which trifurcates proximally to the petal base. The median trace supplies the petal and the two laterals supply the stamen rows. The stamen bundles divide radially in their upward course through the filament tube to supply the stamen pairs and are depleted supplying the uppermost pairs. The five whorled, alternipetalous gynoecium primordia are initiated more or less concurrently. The individual style primordia fuse postgenitally to form a compound style. The floral meristem is consumed during the formation of the five septa, which are formed from the congenital connation of the lateral carpel walls. The phylogenetic implications of the androecium and gynoecium are discussed.

## INTRODUCTION

Floral ontogeny in the Malvaceae has attracted attention due to the presence of some unusual floral traits, most notably in the androecium. The Malvaceae androecium is monadelphous, the filament tube bearing numerous stamens arranged in pairs. The stamens or stamen pairs are organized into ten rows or fascicles, and are bisporangiate, in contrast to tetrasporangiate in most angiosperms.

The traditional theory of the angiosperm flower visualizes it to be a modified shoot. According to this hypothesis, stamens are sporophylls which bear marginal sporangia and are homologous with foliage leaves. In many Ranales for example, the microsporangia are borne on broad, leaf-like structures instead of on filaments (Canright, 1952). It is difficult to reconcile the fasciculate Malvaceae androecium in terms of this classical flower theory. Van Heel (1966) pointed out the conceptual difficulties that are required to hypothesize stamens occurring in pairs in order to account for the stamen fascicles. The androecium in the Malvaceae has instead been used as evidence for alternate theories of stamen phylogeny. Wilson (1937, 1942), a proponent of the telome theory, thought the Malvaceae androecium developed from the reduction and fusion of a dichotomously branched system bearing terminal sporangia. The bisporangiate stamens would be a manifestation of the fusion of two adjacent sporangia. The stamen pairs represent penultimate dichotomies of two adjoining sporangia pairs that have failed to unite. Presumptive further reduction and fusion of the two bisporangiate members of the stamen pair would result in the prevailing tetrasporangiate angiosperm stamen. Melville (1963) also considered the bisporangiate stamen in the Malvaceae to be an original and not a derived character. Van Heel (1966, 1969) thought the Malvaceae androecium to be telomic in origin. However, unlike Wilson (1937) and

Melville (1963), van Heel considered the individual members of the stamen pairs to be "half-stamens," phylogenetically derived from the splitting of a tetrasporangiate stamen. Other authors also felt that the fascicles of bisporangiate stamens in the Malvaceae arose as a result of the subdivision (chorisis or dedoublement) of individual stamens to form many (Saunders, 1936, Rao, 1952). Melville (1963) attempted to reconcile the classical and telomic theories in his gonophyll theory, in which a stamen fascicle is derived from a terminal leaf (androphyll) bearing a fertile microsporangial branch.

Although not as extensive as androecial development, gynoecial development in the Malvaceae has also been studied. Saunders (1936) observed that there are apparently five locules but ten stylar branches in the mature flowers of several species in the tribe Ureneae, Malvaceae. She contended that the gynoecia of these species are composed of five "fertile carpels" (composed of the ovule-bearing septa) and five "sterile carpels" (composed of the locules and their walls). In Saunders' theory of carpel polymorphism, normally only "sterile carpels" bear styles, but that in these malvaceous species both carpel types bear styles. Her interpretations of the malvaceous gynoecium were refuted by van Heel (1978). He demonstrated that ten carpels are initiated, but five of the carpels do not bear ovules. The locules of the sterile carpels are visible in early stages of development, but are subsequently obliterated during

later development of the five fertile carpels. The stylar branches of the five non-ovule bearing carpels are retained in the mature flower.

Numerous Malvaceae species bear only one ovule per locule. In Urena lobata and Pavonia praemorsa, the solitary ovules appear to arise on the floral apex opposite the developing carpels, and not on the flanks of the carpels themselves (van Heel, 1978). Van Heel felt that the classical theory of a conduplicate carpel with marginal placentation could not be applied in these cases. He concurred with Sattler (1974) that in these situations a non-ovule bearing carpel should be named by a neutral term such as gynoecial appendage, as the classical floral theory defines the carpel to be an ovule-bearing structure.

Hibiscus is reported to be the largest genus of the family, comprising approximately 250 species (Bates, 1965). Despite its size and morphological diversity, the floral ontogeny of only a few Hibiscus species has been reported. We report here the floral ontogeny of Hibiscus acetosella Welw. ex Hiern, a previously unexamined species. This study will also serve as a basis for the ontogenetic examination of abnormal H. acetosella flowers produced on plants derived from tissue culture.

## MATERIALS AND METHODS

Flower buds in various developmental stages collected from field and greenhouse grown plants at Louisiana State

University, Baton Rouge, were fixed in FAA (aqueous solution of 10% formalin - 5% acetic acid - 50% ethanol) for three to seven days. After fixation, buds were stored in 70% ethanol. Materials for scanning electron microscopy (SEM) were further dehydrated in 95% ethanol for a minimum of four hours. This protocol leaves plant tissues brittle, which facilitated the subsequent dissections performed in 95% ethanol under a compound dissecting microscope. Material for SEM was further dehydrated through an absolute ethanol-acetone series, critical point dried, mounted on aluminum stubs with Television Tube Koat (tm-GC Electronics), and coated with 370A gold-palladium in an Edwards S150 Sputter Coater. Specimens were examined with a Hitachi S-500 Scanning Electron Microscope at an accelerating voltage of 25 kV.

Plant materials for light microscopy were removed from the 70% ethanol storage and dehydrated in a tertiary-butyl alcohol series. Materials were embedded in Paraplast-Plus paraffin, sectioned at 5 to 7 um, and stained in either 1% alcian blue - 1% safranin stain schedule or in 0.05% toluidine blue. Stained sections were photographed on a Zeiss Photomicroscope using Kodak Technical Pan Film 2415 (ASA 125) and a Kodak Wratten Filter No. 11.

## TERMINOLOGY

Several sets of terminology have been employed to describe floral organogenesis. Described here is the

terminology employed in this report. The saggital median plane is the longitudinal plane going through the center of the vegetative axis bearing the flower and through the subtending leaf below that flower. The abaxial side of the flower or of an organ is the side away from the vegetative or flower axis respectively; the adaxial side is the side adjacent to the axis. Petal aestivation is the pattern of petal overlap in the flower bud prior to anthesis. Style primordia refer to the individual styles prior to their fusion. Stylodes refer to the free style branches above the fused region of the compound style. Postgenital fusion of organs refers to surface fusion observable during ontogeny, or fusion that occurs after organ initiation. Organs initiated as a unit structure are referred to as connate and so presumably arise from zonal growth (after Cusick, 1966); the frequent use of "phylogenetic fusion" or "congenital fusion" in this context will be discussed later. The numerical ordering of the successive androecial primordia used here is the same as that adopted by Sattler (1973) for Althaea rosea. The individual bisporangiate stamens are referred to as unit stamens after Sattler (1973), not as "half-stamens" as referred to by Saunders (1936) and van Heel (1966).

## RESULTS

Organography. The flowers of H. acetosella are borne solitary in the leaf axils on both the primary vegetative

axes and on short axillary shoots. Each flower bears seven to ten free bracteoles which are adnate to the calyx base. The calyx is composed of five sepals which are free for over half their length, forming a connate tube at their base. The calyx is valvate in bud. The five alternisepalous petals are basally adnate to the androecial tube and convolute in bud. The androecium is monadelphous, the bisporangiate stamens occur either in pairs or singly on short filaments arising from the tube. The stamens are distributed in ten vertical rows or fascicles, two rows opposite each petal, with the individual stamens distributed the entire length of the androecial tube. The tube is terminated by five small antepetalous teeth. The five alternipetalous carpels are connate in a compound ovary, each of the five locules containing five or six bitegmic ovules. The style is terminated by five capitate stigmas on short, spreading stylodes. The fruit is a loculicidal capsule. The bracteoles and calyx are persistent; whereas, the corolla, androecium, and style all wither and fall off several days after anthesis.

Organogenesis. The vegetative meristem initiates alternating leaves and stipule - pair primordia. When the day length is appropriate (12 hours or less) floral meristems arise subsequently in the leaf axils (Figs. 1, 2). A concomitant reduction in stem elongation accompanies floral initiation.

In early development, the floral meristem appears convex when viewed abaxially (Figs. 3). The floral meristem shortly becomes truncate viewed abaxially (Fig. 4) and circular viewed from above (Fig. 6) as the bracteole primordia are initiated. The first bracteole appears on the adaxial flank of the floral meristem (Fig. 5). Other bracteole primordia are initiated in a helical order (Figs. 6, 7) until seven to ten bracteoles are formed. A pronounced groove develops between the bracteoles and the floral apex resulting from bracteole initiation and floral meristem growth (Figs. 6, 7).

The calyx primordia arise concurrently with or shortly after initiation of the last bracteole. Five sepal primordia appear equidistantly spaced on the apex periphery (Fig. 8). A connate tube forms shortly afterwards at their base (Figs. 9, 11). The remainder of the floral apex is pentamerous in shape after initiation of the calyx tube (Fig. 10). The sepal lobes are carried upward from zonal growth in the calyx tube, and shortly arch over the remainder of the floral apex (Fig. 11). The margins of the sepal lobes eventually meet, culminating in valvate aestivation in bud (Figs. 12, 13). Sepal lobes account for about two-thirds of calyx height with the remainder comprised of calyx tube in the mature bud. Trichome initiation begins distally on the sepal lobes (Fig. 11) and proceeds basipetally (Fig. 14) until the entire surface of the lobes and tube exhibit them (Fig. 15).

Initiation of the petal primordia coincides with the overarching growth of the calyx lobes. The petal primordia arise in alternisepalous positions at the points of the pentamerous floral apex (Fig. 16). A ring meristem shortly arises (Fig. 17), the petal primordia low on the outer flank of the ring meristem (Figs. 18, 19). The early petal primordia are asymmetrical in shape (Fig. 20), and as development continues each petal becomes oblique in its orientation with one margin canted inward, the other outward (Fig. 25). This determines the direction (clockwise or counterclockwise) of petal aestivation, as the outward-canted margins become the overlapping petal edges in bud. (Figs. 25, 27, 28). Growth of the petal primordia is delayed until after anther differentiation. Petals expand laterally (Fig. 27) then curve inwards over the flower apex, becoming convolute in the bud (Fig. 28). The petals are basally adnate to the filament tube (Figs. 35, 36) due to zonal growth below the point of attachment of the organs.

Faint antesepalous radial depressions in the ring meristem demarcates the five antepetalous primary androecial primordia (Figs. 17, 18) shortly after petal initiation. Five antepetalous oblique radial grooves appear as the five primary androecial primordia unevenly divide laterally to form five pairs of secondary androecial primordia (Fig. 20). The elliptical secondary androecial primordia in turn divide radially, forming a total of 20 tertiary androecial primordia, four opposite each petal (Figs. 21, 22).

Tertiary primordia centrifugally bifurcate (Figs. 23, 24) to form 40 quaternary primordia which are organized as slightly-stalked pairs (Figs. 25, 26). The quaternary primordia develop into stamens-pairs, each stamen bisporangiate with the sporangia laterally oriented (Fig. 27). Stamen-pair filaments are basally connate above their point of attachment to the filament tube (Fig. 31). One to three additional androecial primordia are initiated in line with each of the two antipetalous rows of primordia in an alternating centrifugal sequence. The initiation of these primordia varies slightly, becoming visible at some time during the division of the tertiary primordia (Figs. 23 - 26). These additional primordia may divide and develop into a stamen pair, develop into a single stamen (Figs. 29, 30), or form a filament without sporangia (Fig. 30). Single stamens and the sterile filaments were observed to occur at the base of the androecium, below the paired anthers, adjacent to petal bases. Stamen number varied, ranging from 59 to 73 in ten flowers examined at anthesis.

Stamens are organized vertically into six to ten whorls and laterally into two rows opposite each petal. Whorls result from the concurrent divisions of the androecial primordia (Figs. 20, 21, 23, 25). Whorl identity is lost in intermediate stages of development as the stamen pairs become physically crowded. Stamen whorls become discernible again at anthesis as the androecial tube elongates, eliminating crowding.

The two rows of stamens opposite each petal are termed major and minor rows (van Heel, 1966). Row position can be predicted by division of the primary androecial primordia, the larger division product becoming the major row. The major row always bears the uppermost stamen pair on the androecium tube, the successively lower stamen pairs or stamens of the two rows alternating as the teeth of a zipper (Figs. 29, 30), and is opposite the overlapping petal. The major row bears the same as, one more, or two more stamens than the minor row. If the two rows bear a pair and a single stamen respectively at their base, the former will then finish the major row; if a single stamen and a sterile filament are in the basal positions, then the stamen again will be in the major row (Fig. 30). Viewed abaxially, the major row may be to either the left or right side, just as petal convolution may be clockwise or counterclockwise.

Androecial tube elongation occurs predominantly after sporangia formation. The stamens are distributed equally over most of the tube, with a small region free of stamens below the insertion of the basal stamens and above the petals. The tube is terminated by five small sterile teeth (Figs. 30, 32), which are antepetalous in position. The teeth are not readily visible in the mature flower due to their small size and masking by the terminal stamens.

Five whorled, alternipetalous gynoecial primordia are initiated at about the time of division of the tertiary androecium primordia. The primordia arise as five

equidistant mounds on the periphery of the slightly concave apex to the inside of the androecium tube (Figs. 37, 38). A connate gynoecial tube forms below the five free apical lobes (Figs. 39 - 42). The apical lobes develop to form the stylar primordia. As stylar lobes overarch the apex they become appressed, first laterally, then also radially (Figs. 40 - 42), ultimately fusing into a compound style. The external lines of fusion are evident in young material (Fig. 42) but become less visible as the flower bud matures (Figs. 43 - 45). Internal fusion lines also become less distinct with time, as protodermal cells of the opposing primordia divide periclinally (Figs. 46, 47). The distal region of the compound style extends beyond the apex of the androecial tube at anthesis, the five centrifugally radiating stylodes each terminated by a discoid stigma.

The inward developing carpel margins become visible shortly after initiation of the stylar primordia (Figs. 37, 38). The locules are formed under the overarching style primordia from the lateral and centripetal expansions of the carpel margins (Figs. 38 - 40). The lateral walls of the carpels are connate (Figs. 38, 40, 49). Concrescence of the ventral carpel margins results from the continued lateral development of the connate carpel margins (Figs. 40, 49). Carpel development consumes the entire floral meristem (Figs. 36, 48). The funiculi of each ovule are attached to the ventral carpellary margins. The ovules are initiated at about the time of the concrescence of the carpel margins.

The ovules are crassinucellate, bitegmic, number five to six per locule, and are borne on the placentae in an alternating sequence. The basal ovules are the first initiated (Figs. 36, 48).

Flower Vascularization. This study was conducted to compare floral morphology in wild type and tissue culture-derived H. acetosella plants. A complete anatomical comparison of the two is to be presented at a later date. For our current needs, only a limited amount of material was examined to compare with the reports in the literature on floral vascularization within the Malvaceae. We report here a preliminary analysis of the early vascularization of the bracteoles, calyx, corolla, and androecium. The gynoecial vascularization was not elucidated, as this was not sufficiently developed in the flower buds examined. The pedicel stele divides into nine collateral traces slightly below the bases of the bracteoles. Three of the traces are larger than the other five, which are located as two pairs and a single between the former. The three larger traces diverge at the bases of the bracteoles, forming a transverse ring that rapidly divides into twice as many traces as there are bracteoles. Every other trace becomes a median bracteole bundle. All but five of the other traces separate to form the lateral bundles of adjacent bracteoles. Each of the other five traces trifurcates into three strands, the outer two becoming lateral bundles of adjacent bracteoles.

The five bundles remaining in the stele and the five outer bundles remaining after the supplying of the bracteoles ramify below the sepal bases, forming a ring of vascular tissue to the inside that goes on to supply the carpels, and 10 bundles in a ring to the outside. The five alternating traces formed from the ramification of the stelar bundles lie on sepal radii and supply the median sepal nerves. The remaining five bundles radially branch at the base of the sepals. The five strands formed to the outside become the lateral sepal bundles. These bifurcate just below the termination of the calyx tube and form the lateral bundles of each of the calyx lobes. The free calyx lobes then are supplied by one median and two lateral strands.

The five inner bundles formed from the branching strands at the lateral sepal bases continue upwards to just below the androecial tube and petals. The strands separate tangentially, forming a larger central strand and two lateral strands. The five central strands, lying on petal radii, radiate slightly outwards and ramify at the petal bases to supply the numerous petal veins. The ten lateral strands continue upwards into the androecial tube, supplying the ten stamen fascicles (Fig. 33, 35). The strands branch radially below the insertion on the filament tube of the stamens or stamen-pairs, supplying each single or paired stamen with a single trace. The strand to a stamen pair branches at the common filament base, supplying a single

trace to each of the free anthers (Fig. 34). The ten fascicle traces become exhausted, the distal ends radiating outward to supply the uppermost stamen-pair of each row. The androecium strands remain on petal radii throughout their upward path. The five teeth at the apex of the androecial tube are not vascularized.

## DISCUSSION

Ontogenetic studies in the Malvaceae date back to Payer (1857), the first to describe floral development in the family. Examinations of the malvaceous androecium include Saunders (1936), Wilson (1937), Rao (1952), Melville (1963), and van Heel (1966, 1969). The malvaceous gynoecium has been discussed in Saunders (1936), Klotz (1975), and van Heel (1978). Other ontogenetic examinations in the Malvaceae include Joshi, Wadhwani, and Johri (1967) and Sattler (1973). Hibiscus species cited in these studies include H. ilicifolius (Wilson, 1937), H. pedunculatus (Melville, 1963), H. micranthus, H. solandra (Rao, 1952), H. rosa-sinensis, H. syriacus, and H. trionum (Saunders, 1936), H. campylosiphon, H. micranthus, H. pulvinar, H. rosa-sinensis, H. syriacus, and H. trionum (van Heel, 1966), and H. ilicifolius and H. syriacus (Payer 1857). Hibiscus acetosella has not been previously examined.

The floral organs of H. acetosella are initiated in centripetal sequence. Calyx, corolla, androecium, and gynoecium primordia are initiated simultaneously within

whorls. The corolla and androecium arise from a ring meristem. The androecium primordia are initiated centrifugally. The general organogenesis of H. acetosella is in agreement with that of other Malvaceae species (van Heel, 1966, Sattler, 1973).

Petal aestivation. Petal aestivation in H. acetosella was found to be regular, with 48 of 51 flowers examined convolute. Petal aestivation in other Malvaceae is either convolute or various imbricate patterns (van Heel, 1966, Sattler, 1973). Aestivation was either clockwise or counterclockwise, the direction appearing to be randomly selected. Tucker (1984) considered petal aestivation to be a mid-developmental feature in the Leguminosae and perhaps also in other taxa. In H. acetosella, it appears that petal aestivation is determined early in development. Petal aestivation in H. acetosella is due to the oblique insertion of the petal primordia and the inward and outward canting of their respective margins. Aestivation is determined well before the edges of adjacent petals overlap, and can in fact be predicted by the uneven division of the primary androecium primordia, the larger division product located opposite the eventual overlapping petal edge.

Androecial development. Centrifugal stamen initiation was first reported in the Malvaceae by Payer (1857). Corner (1946) reported centrifugal stamens to be present in 13

families. A more recent survey listed 32 families (Tucker, 1972). Uhl and Moore (1977, 1980) reported centrifugal stamens in the Arecaceae. Stamen initiation is centrifugal or centrifugal intergraded with lateral in all Malvaceae reported to date (Payer, 1857, Saunders, 1936, Rao, 1952, van Heel, 1966, Sattler, 1973). This is also true of Hibiscus acetosella, as reported here.

Corner (1946) felt that the centrifugal androecium was a most important systematic character that could define plant families as a natural phylum. Cronquist (1968) also gave systematic importance to the direction of stamen initiation. Sattler (1972) questioned the merit of lending one trait such systematic weight. Pauze and Sattler (1978) reported stamen initiation to be centripetal in Ochna atropurpurea, (a species from Cronquist's centrifugal branch), and also listed other examples of centripetal and centrifugal stamen initiation from the same families. The direction of stamen initiation in separating taxa may be useful at certain levels, but its use as a criterion to separate major phyla of angiosperms is questionable.

Each longitudinal row of stamens in H. acetosella is vascularized by a single bundle that traverses the filament tube in line with the stamens. The stamen rows are equivalent to stamen fascicles according to Wilson (1937), who defines a fascicle as any cluster of stamens vascularized by a common trace. Corner (1946) and Tucker (1972) indicated that there is a correlation of

centrifugality and a fasciculate arrangement of stamens. The stamen fascicle traces may be equivalent to trunk bundles, which Tucker (1972) defines as a receptacular bundle that supplies a stamen fascicle and no other organ. After divergence of the common petal-stamen traces in H. acetosella, the stamen fascicle traces supply no other organs than the stamens. Though this pattern of vascularization has been reported in all other Malvaceae examined (Wilson, 1937, Rao, 1952, van Heel, 1966), the stamens of the Malvaceae are generally not referred to as fasciculate (van Heel, 1966, Tucker, 1972). This is in part due to the fact that in many Malvaceae the stamens in the mature flower physically crowd one another, so discrete clusters or rows of stamens are not apparent. In H. acetosella, the rows of stamens are visible at all stages of development and so are considered fasciculate.

There have been several interpretations of the organization of the stamens in the Malvaceae. Saunders (1936) and Rao (1952) regarded the Malvaceae stamens to be arranged in ten obdiplostemonous groups, the inner whorl of stamens having completely disappeared. Melville (1963) and Van Heel (1966) felt it more logical to assume from the conjoint nature of the vascular bundles the presence of five laterally fused groups, each composed of a petal and opposing pair of stamen rows. Saunders (1936) reported that the "half" (bisporangiate) stamens of the Malvaceae arose from the divergence of the median petal trace splitting or

"halving" the stamen row traces. Her interpretation has been sharply disputed (Rao, 1952, van Heel, 1966), especially in her belief that external form can be determined by the action of vascular tissue. Van Heel (1966, 1969) felt that the pairing of the bithecous stamens was evidence for a phylogenetic superficial splitting of a tetrasporangiate stamen into two half stamens. Both Wilson (1937) and Melville (1963) felt the bisporangiate stamen in the Malvaceae was a basic, not derived condition. Wilson (1937, 1942), Melville (1963), and van Heel (1966, 1969) believed the pairing of the stamens and the vascular condition of the Malvaceae androecium was evidence for derivation from a primitive dichotomously branched system bearing terminal male sporangia. Wilson used this evidence in support of the telome theory; Melville used it in favor of his gonophyll theory of stamen phylogeny. The anatomy of the Malvaceae androecium is difficult to reconcile with the classical theory of stamen derivation from a foliar form (Puri, 1951, van Heel, 1966).

The stamen rows in H. acetosella can be termed major and minor rows, as did van Heel (1966) for other Malvaceae. The row in front of the overlapping petal edge is always the major row. The rows can be predicted by the uneven division of the primary androecium primordia, the larger division product becoming the major row. The major row contains as many as or more stamens than the minor row, both in our species and in other Malvaceae. Van Heel (1966) stated only

that there were more stamens in the major row due to "more room between the floral centre and the further outwards inserted overlapping petal halves." This statement does not make clear whether van Heel meant that the lack of mechanical restriction allowed more primordia to differentiate, or that the larger volume of floral meristem in this region could form more primordia. Mechanical pressure has been shown to determine the shape of the floral apex in various palms, which in turn influenced the number and position of stamen primordia (Uhl and Moore, 1977, 1980, Uhl and Dransfield, 1984). This pressure was exerted by the extremely fibrous bracts and perianth surrounding the floral apex. It seems unlikely that similar mechanical pressures are being exerted in H. acetosella, as the bracteoles and perianth parts are not fibrous, and the corolla primordia remain small throughout stamen initiation. It is more likely that the oblique insertion of the petal primordia on the flank of the petal-stamen ring primordium has simply left more meristematic tissue available to form stamen primordia.

Gynoecial development. In examinations of floral "fusions" it is important to examine flowers at all stages of development, not just mature flowers. This is most evident in H. acetosella. The five style primordia are clearly initiated as distinct primordia, but become fused with time. The lines of fusion are distinct early on, but periclinal

divisions in the epidermal and subdermal layers of the appressed surfaces masks the sites of fusion, such that in the mature flower it would be difficult to infer the occurrence of postgenital (during development) fusion. This led to the misleading impression that postgenital fusions were rare until the work by Baum (1948) demonstrated the relative frequency of such events. Postgenital fusion of the style primordia has been demonstrated in other Malvaceae (Sattler, 1973, Klotz, 1975, van Heel, 1978).

The terminology concerning floral "fusions" has been somewhat ambiguous. Fusion during ontogeny has traditionally been known as ontogenetic or postgenital fusion (Cusick, 1966). Sattler (1978) recommended the discarding of the general term "ontogenetic fusion" in favor of "surface fusion" and "meristem fusion", for the two recognized processes (by Sattler) of fusion during ontogeny. Sattler also recommended the use of "postgenital fusion" only in the sense of surface fusion, or replaced by surface fusion. In H. acetosella, we refer to the demonstrable surface fusion of the style primordia as postgenital fusion.

The formation of phylogenetically connate organs has variously been labeled as congenital or phylogenetic fusion (Cusick, 1966). There have been serious objections raised to using "fusion" in a phylogenetic sense, as it implies the merging of discrete entities, an action which cannot be readily observed. To avoid implying false phylogenetic origins, Sattler (1978) proposed using the neutral term

"continuity". Continuity can be used in any reference to union of parts, whether observable (postgenital) or inferred (phylogenetic). Cusick (1966) proposed the term "zonal growth" for the upward growth of floral organs as a unit structure. The peripheral ovary wall, septa, and locules of H. acetosella are simultaneously brought into relief during gynoecial ontogeny. There is no evidence of postgenital fusion below the styles at any stage of development. The syncarpous ovary of this species is referred to here as simply connate, arising from zonal growth. Without any developmental information, the compound style and syncarpous ovary would best be referred to as "continuities".

Sattler (1973) and van Heel (1978) describe gynoecium development in several Malvaceae in which only one ovule is formed at the base of each locule. Both interpreted their results to signify that the ovules were initiated on the floral axis, not on the flanks of the carpels, invalidating in their estimation the classical carpel theory. Sattler (1974) argued that many syncarpous superior ovaries could also be interpreted in a similar manner, the ovule-bearing septa being axial rather than carpellate. Gynoecium development in H. acetosella appears to conform to the carpel concept. The floral meristem is exhausted during the formation of the carpels. The multiple ovules per locule are borne in two vertical rows on either side of an ontogenetically closed suture. If this interpretation is correct, it is difficult to accept transfer of the ovules

from a carpellate structure to an axial structure in closely allied taxa as H. acetosella and the Malvaceae species of Sattler (1973) and van Heel (1978). As stated by van Heel (1978), the diversity of gynoecial structures in the Malvaceae warrants further investigation.

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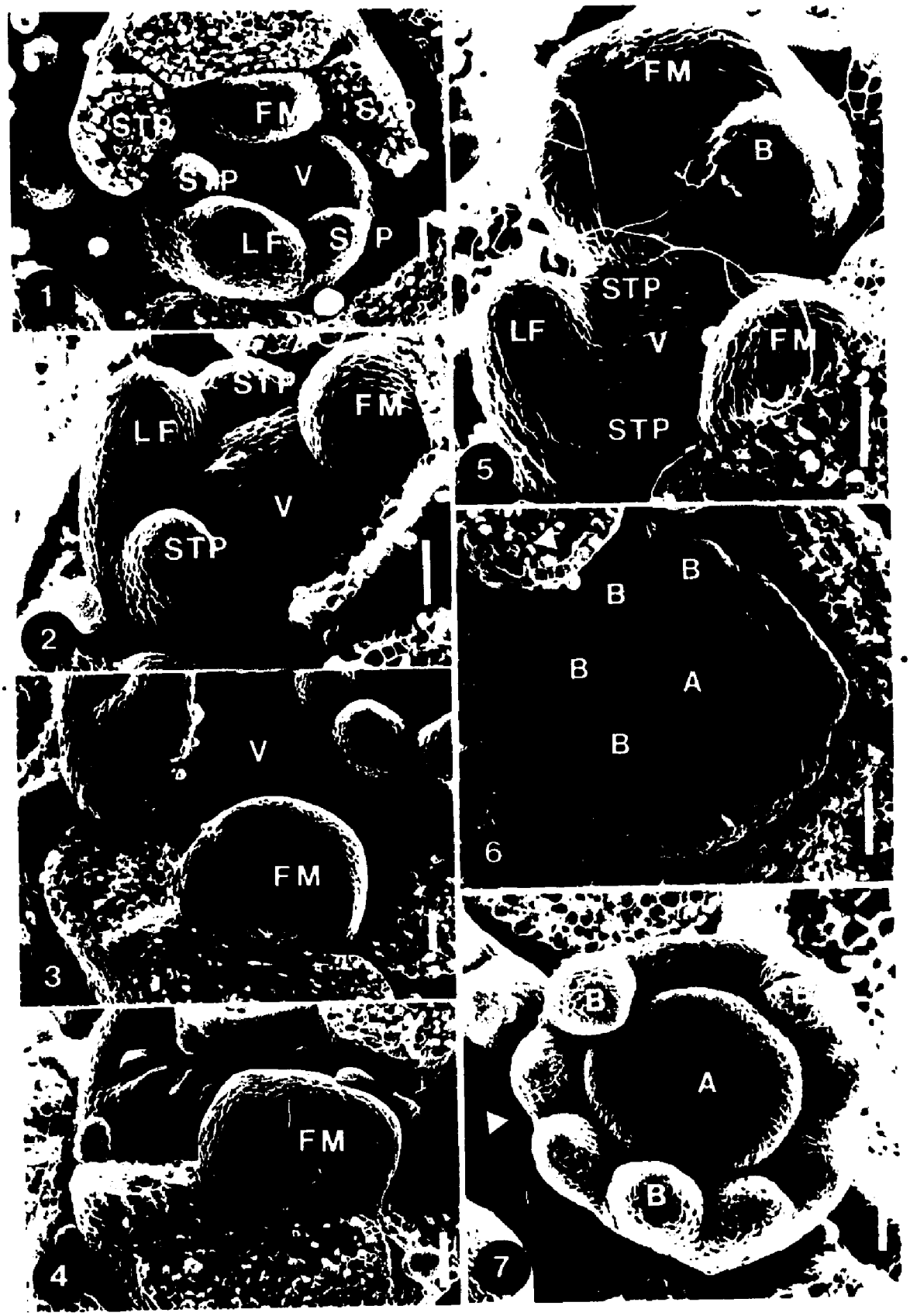
Abbreviations for Figs. 1 - 49. A= floral apex, AT = androecial tube, B = bracteole, CM = carpel margin, CT = calyx tube, F = androecial filament, FM = floral meristem, L = locule, LF = leaf primordia, OB = ovary base, OV = ovule, P = petal, S = androecial primordium (before it becomes anther primordium), SE = sepal, SL = sepal lobe, SP = pollen sporangium, SPS = scar from removal of stamen pair, ST = style or stylode, STP = stipule primordia or scar from removal of stipule, T = terminal tooth of androecial tube, and V = vegetative apex.

Figs. 1 - 7. Floral meristem and early bracteole organogenesis in wild type Hibiscus acetosella. Triangle designates adaxial side of floral primordia. Scale bars = 50 uM. 1. Top view of vegetative apex, with leaf, stipule pair, and axillary floral primordia initiation. Upper leaf and stipule pair removed. 2. Same as 1, lateral view. Arrow designates initiation site of next floral primordium. 3. Lateral view of convex floral primordia. Subtending leaf removed. 4. Lateral view of floral primordia prior to appearance of bracteole primordia. 5. Initiation of first bracteole on adaxial flank of floral primordia. 6. Top view of floral apex bearing four discernible bracteole primordia on the adaxial side. 7. Top view of floral apex with nine bracteole primordia. Helical initiation of bracteoles is inferred from size differences. Bracteole initiation has demarcated the remainder of the floral apex.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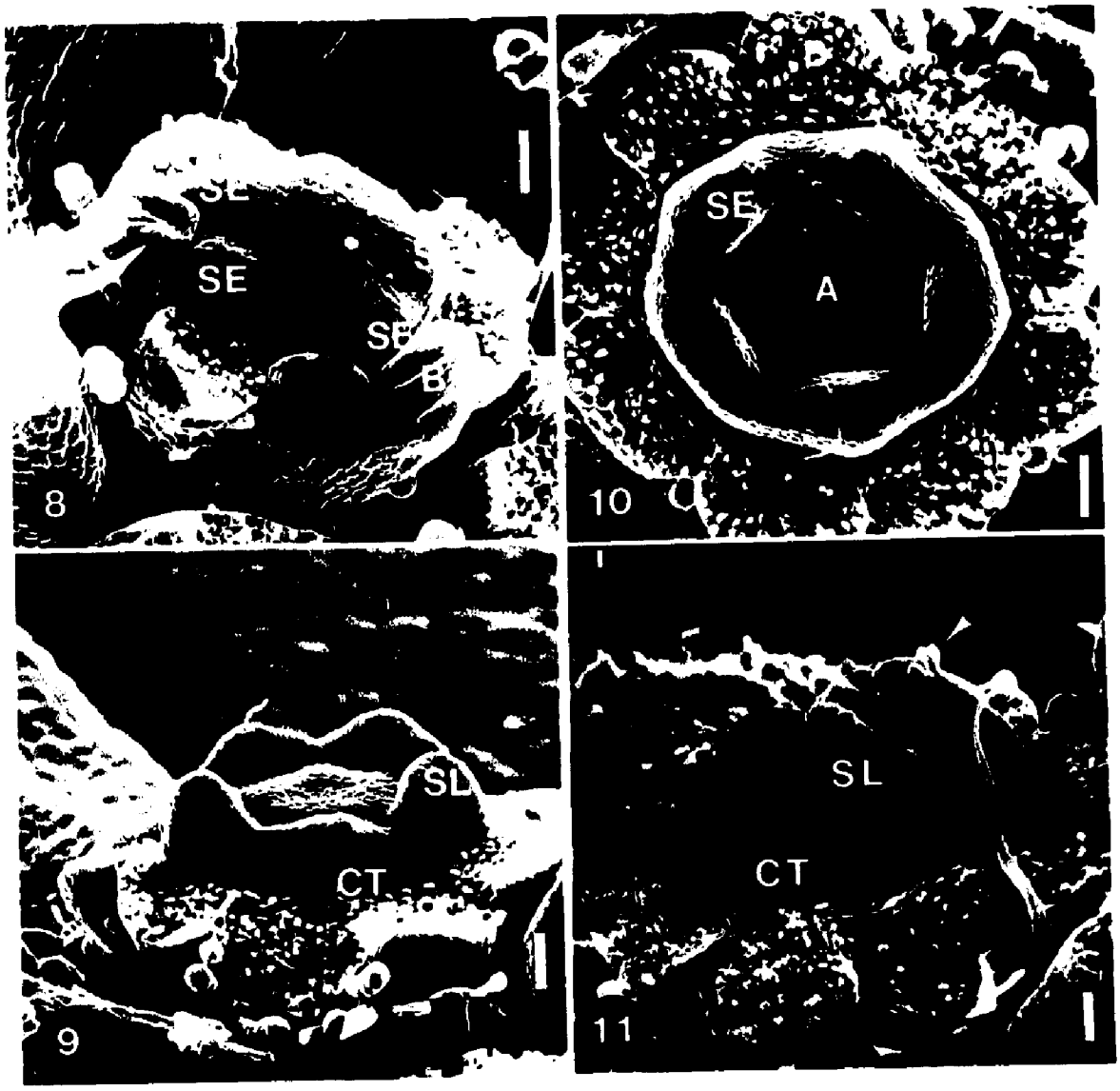
Figs. 8 - 11. Early calyx organogenesis in H. acetosella. Scale bars = 50 uM. 8. Lateral view of initiation of calyx as five separate sepal primordia on periphery of floral apex. 9. Lateral view, early developmental of calyx, showing five sepal lobe primordia atop the calyx tube. 10. Top view of pentamerous calyx after initiation of calyx tube. 11. Lateral view of expanding calyx. The calyx lobes curve centripetally over the floral apex. Note trichome initiation on abaxial and adaxial distal calyx lobe surfaces (arrows).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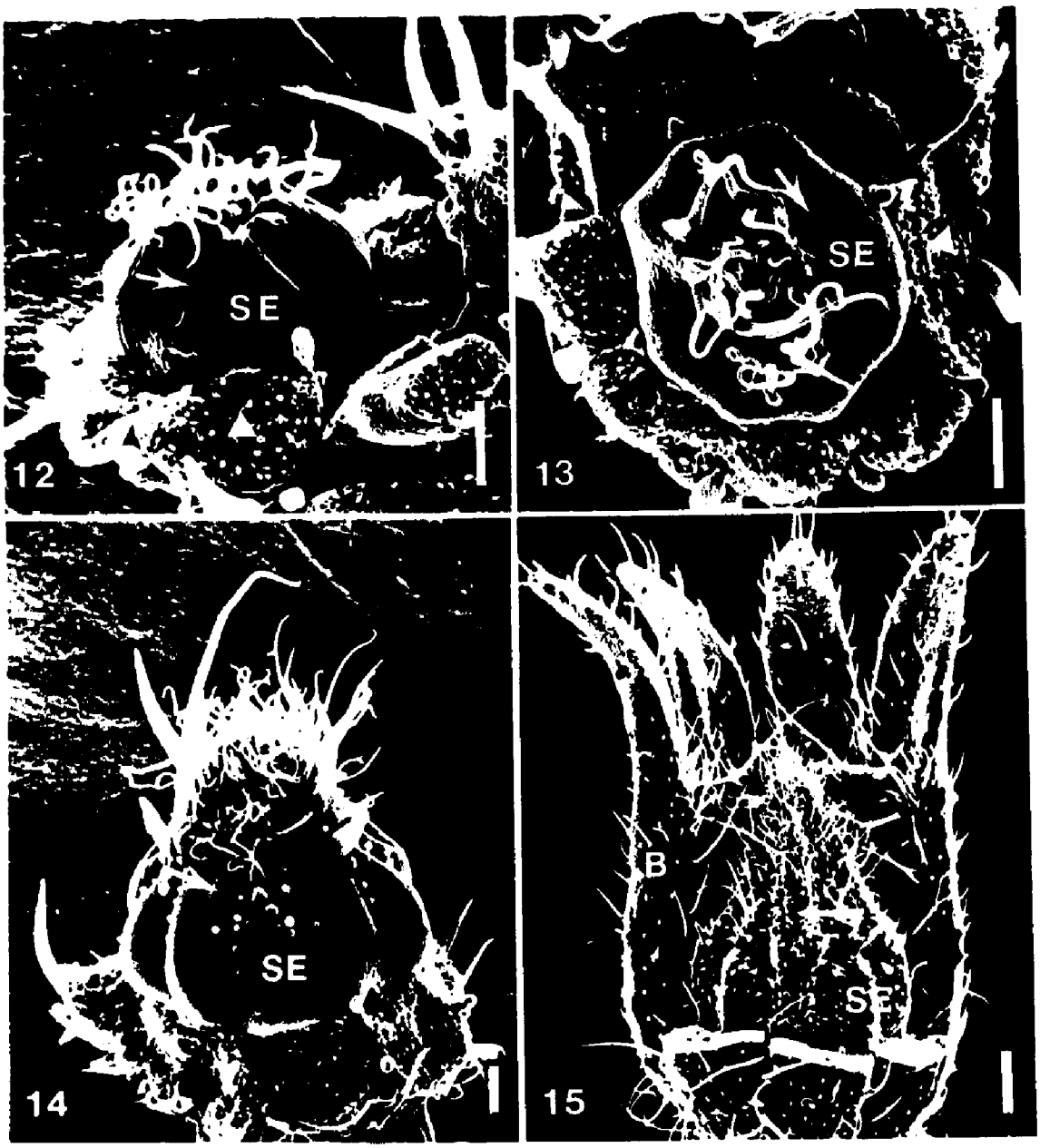
Figs. 12 - 15. Late calyx organogenesis in H. acetosella. Arrows indicate line of appression between adjacent sepal lobes. Triangles mark same side of floral bud in different views. Scale bars = 100 uM for 12 - 14, Scale bar = 500 uM for 15. 12, 13. Lateral and top view, calyx lobes appressed, trichome initiation continuing basipetally. 14. Median and lateral nerves of calyx developed. 15. Calyx covered with trichomes. Line of appression between adjacent sepal lobes still visible.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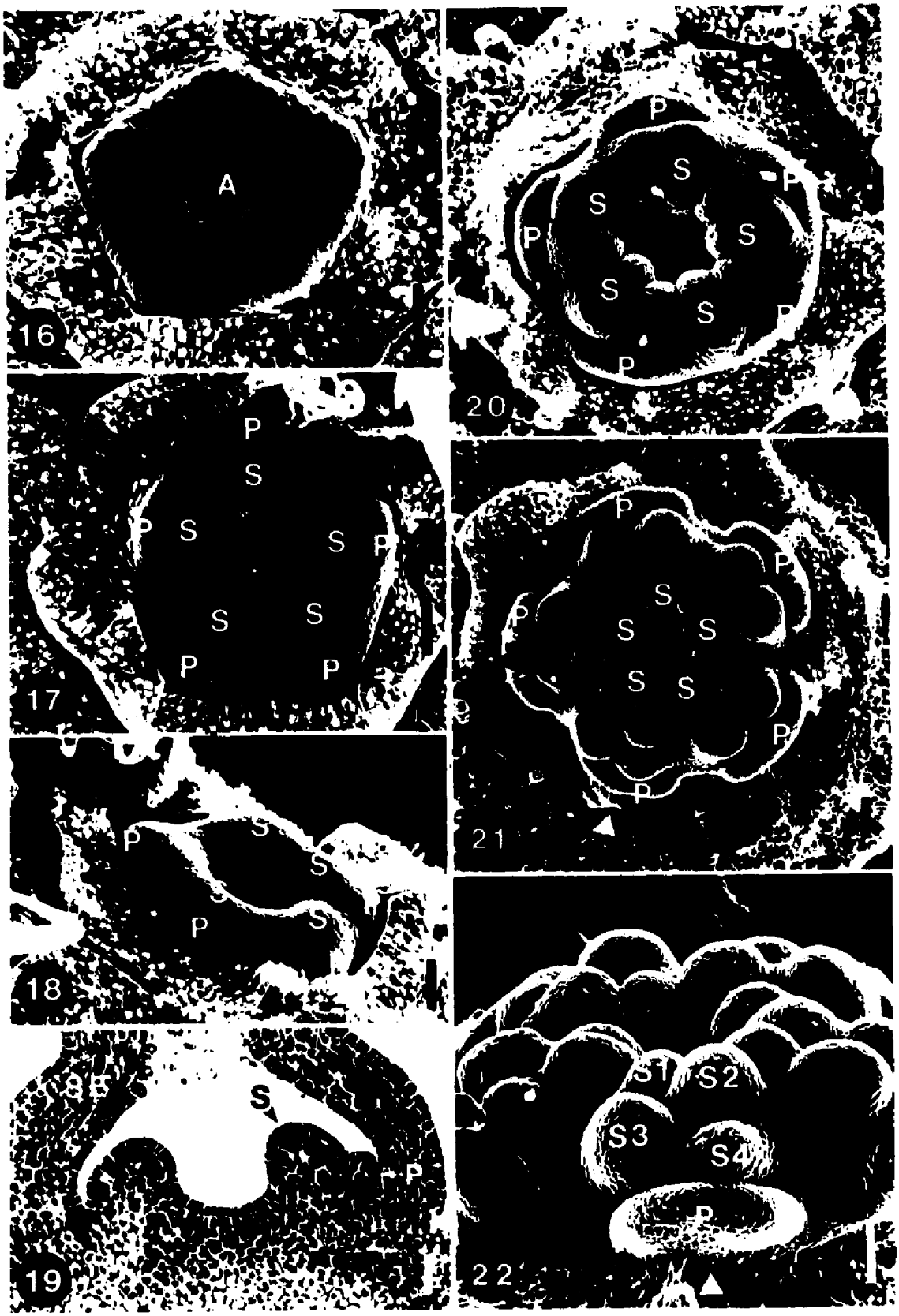
Figs. 16 - 22. Early petal and androecial organogenesis in H. acetosella. Triangles mark same surface in different views. Scale bars = 50 uM. 16. Top view of pentamerous floral apex, slightly concave in center. 17, 18. Top and lateral view of petal and primary androecial primordia initiation. 19. Median section of early petal primordia on abaxial flank of petal androecium ring primordia. Flower meristem is organized in typical tunica-corpus configuration, with two tunica layers. 20. Formation of antepetalous secondary androecial primordia. The larger androecial primordia (labeled) in each antipetalous pair is in the position of the eventual major stamen row. The petal edge adjacent to the major stamen row will be the overlapping edge in the convolute corolla in bud. 21, 22. Top and lateral view of centrifugal initiation of tertiary androecial primordia, all in antipetalous position. The androecial primordia in 22 are numbered in their order of initiation. The tertiary androecial primordia are initiated as four sequential whorls (one whorl labeled in 21), each whorl with five simultaneously formed primordia.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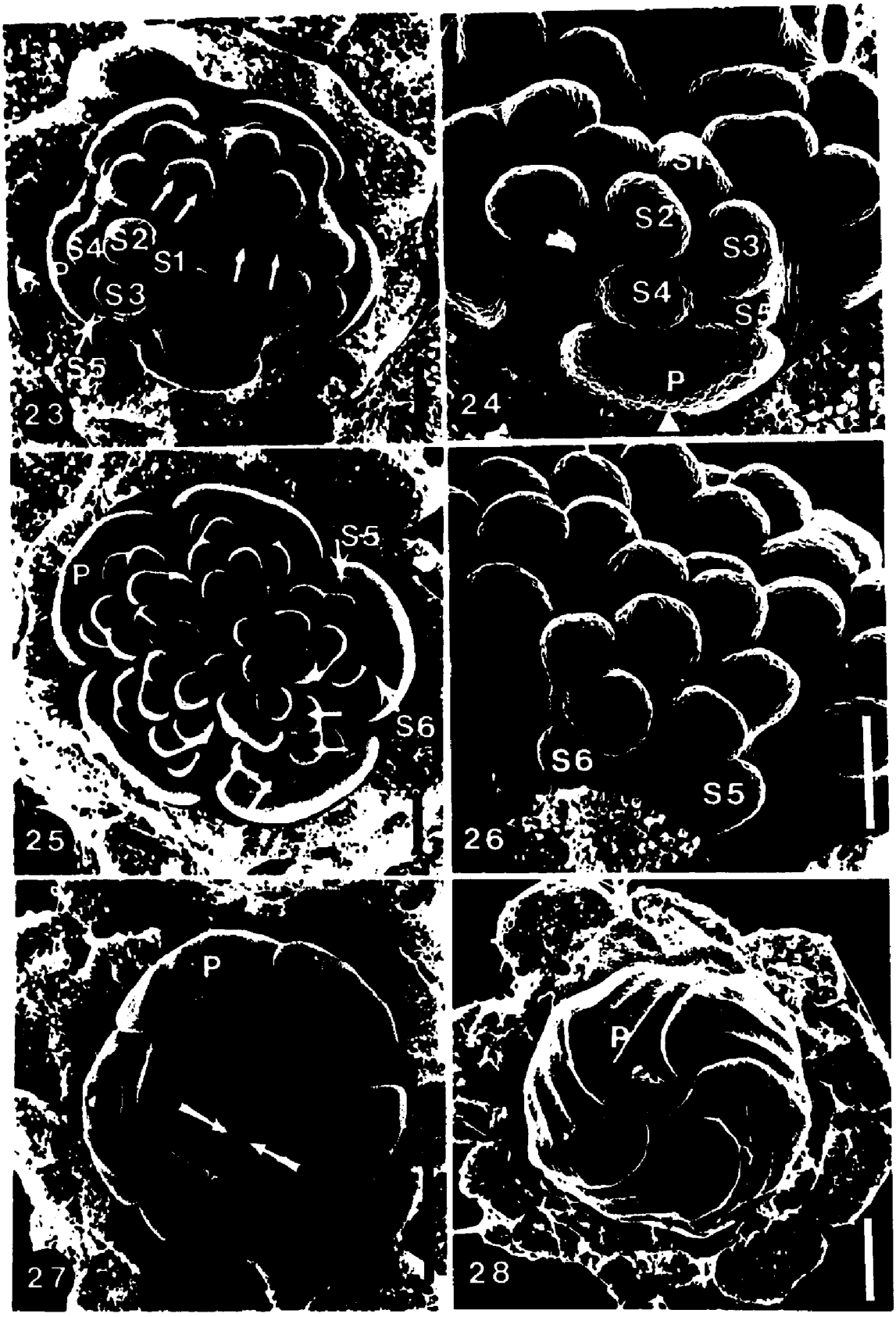
Figs. 23 - 28. Androecial and petal organogenesis in H. acetosella. Triangles mark same petal in different views. Scale bars = 100 uM for 23 - 26, scale bars = 500 uM for 27 and 28. 23, 24. Top and lateral view of the centrifugal initiation of androecial primordia. The labeled primordia (S5 with arrowhead) are numbered in sequence of initiation. The major stamen row contains primordia S1, S3, and S5. Small arrows in 23 mark the formation of quaternary androecial primordia from members of the two inner tertiary androecial primordia whorls. 25, 26. Top and lateral view of quaternary androecial primordia formation and of the centrifugal initiation of androecial primordia in the major stamen row (S5 with arrowhead)) and in the minor stamen row (S6 with arrowhead). Small arrows in 25 mark the formation of quaternary androecial primordia from members of the two outer tertiary androecial primordia whorls. Petal margins overlapping in 25, showing convolute aestivation. 27, 28. Top view of petal aestivation in increasingly mature floral buds. Arrows in 27 indicate the lateral initiation of two anther sporangia.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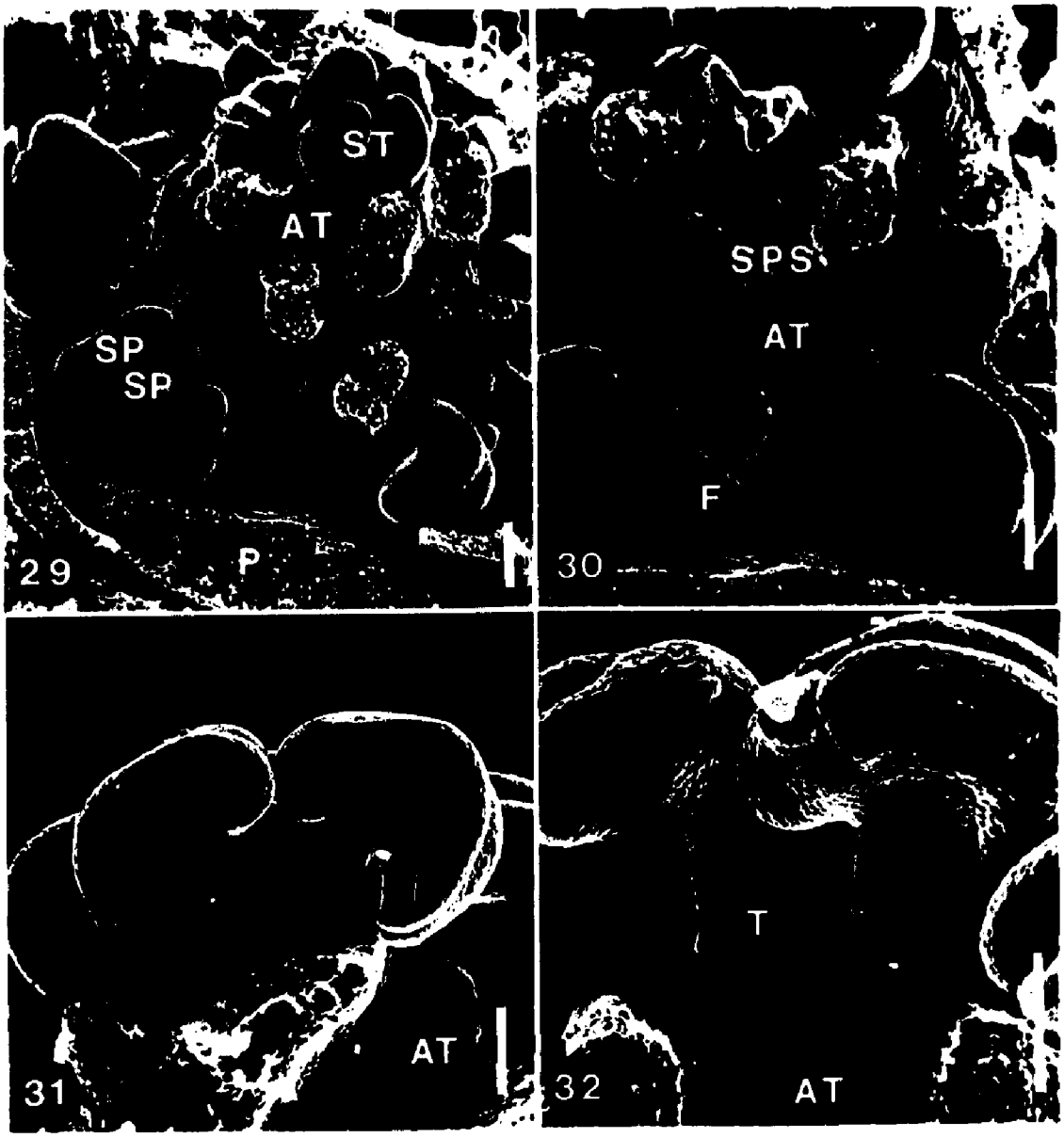
Figs. 29 - 32. Androecial organogenesis in H. acetosella. Scale bars = 150 uM. 29, 30. Lateral view of the major (right) and minor (left) stamen rows in antipetalous positions. The scars on the androecial filament tubes are all from removal of stamen pairs. Note the presence in 30 of a sterile filament (no anther) in the basal position of the minor row. 31. Lateral view of a stamen pair from the distal region of the androecial tube. 32. The adaxial view of one of the five sterile apical teeth of the androecial filament tube.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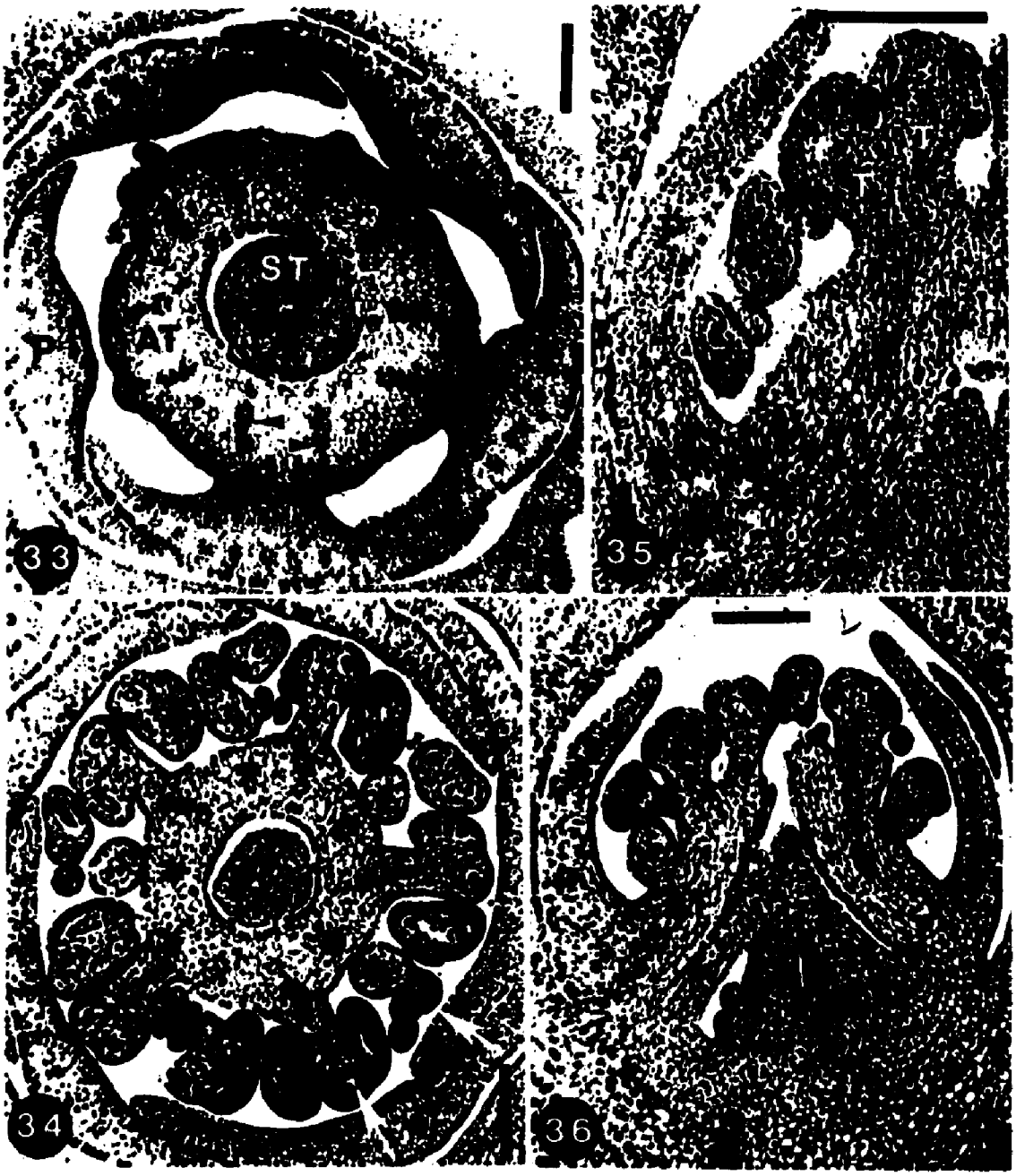
Figs. 33 - 36. Floral anatomy in H. acetosella. Scale bars = 200 uM. 33. Transverse section below the stamen primordia and above the petal adnation to the androecial tube, showing convolute petal aestivation, compound style, and connate filament tube with two procambial traces (arrows) opposite each petal. The traces supply the ten vertical stamen rows. 34. Transverse section slightly higher than in 33, showing the ten vertical rows of stamens and their corresponding procambial traces. The arrows indicate the sites of the two stamen rows (perpindicular to plane of section) which lie opposite the labeled petal. 35. Longitudinal section showing the basal adnation of the petal to the filament tube, and the splitting of the procambial trace to the stamen pair to supply a single trace to each stamen. 36. Longitudinal section showing the basal adnation of the petals to the androecial filament tube, and the depletion of the floral meristem in the formation of the carpels. There are two ovules in the left carpel.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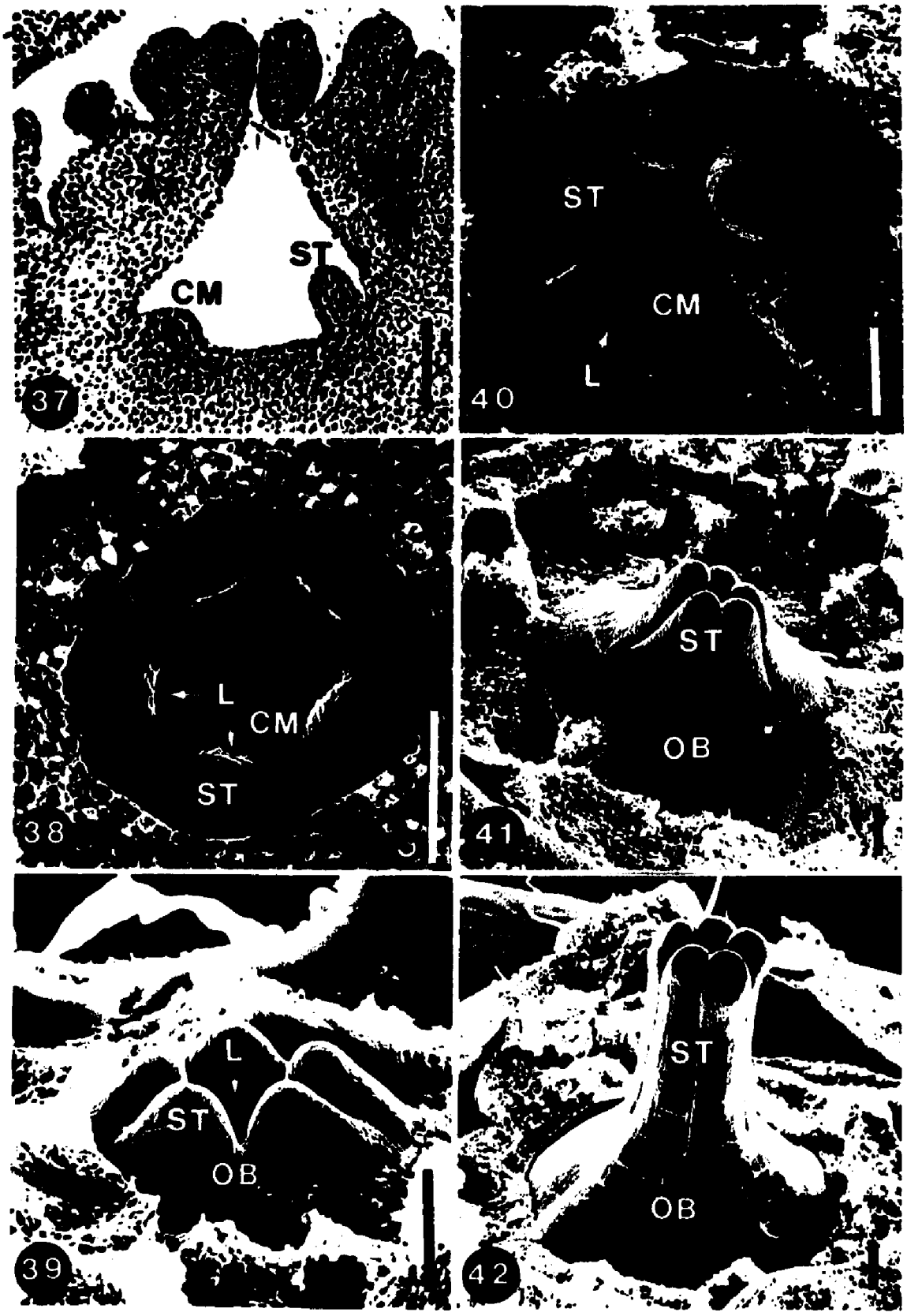
Figs. 37 - 42. Gynoecial ontogeny in H. acetosella. Scale bars = 100 uM. 37. Median section showing early gynoecial development, with a style primordium and a presumptive carpel margin on the periphery of the floral apex. The floral apex is still organized in a two cell-layered tunica, corpus configuration. 38. Top view showing the five style primordia, the inward developing connate carpel margins, and the initiation of the locules underneath the styles. 39. Lateral view showing the free style primordia and the early gynoecial tube. One of the locules can be seen under a style primordia. 40. Top view of a slightly older gynoecial primordia. Two style primordia have been removed to show the connate lateral margins of two adjacent carpels. A small cavity which will become a locule can be seen within one of the carpels. The remaining three style primordia have become laterally appressed. 41, 42. Lateral view of increasingly older gynoecia. The five style primordia have fused, forming compound styles. The lines of fusion between adjacent style primordia are still readily visible. The stigmas have begun to differentiate in 42. The connate ovary base has expanded as the septa and ovules develop internally.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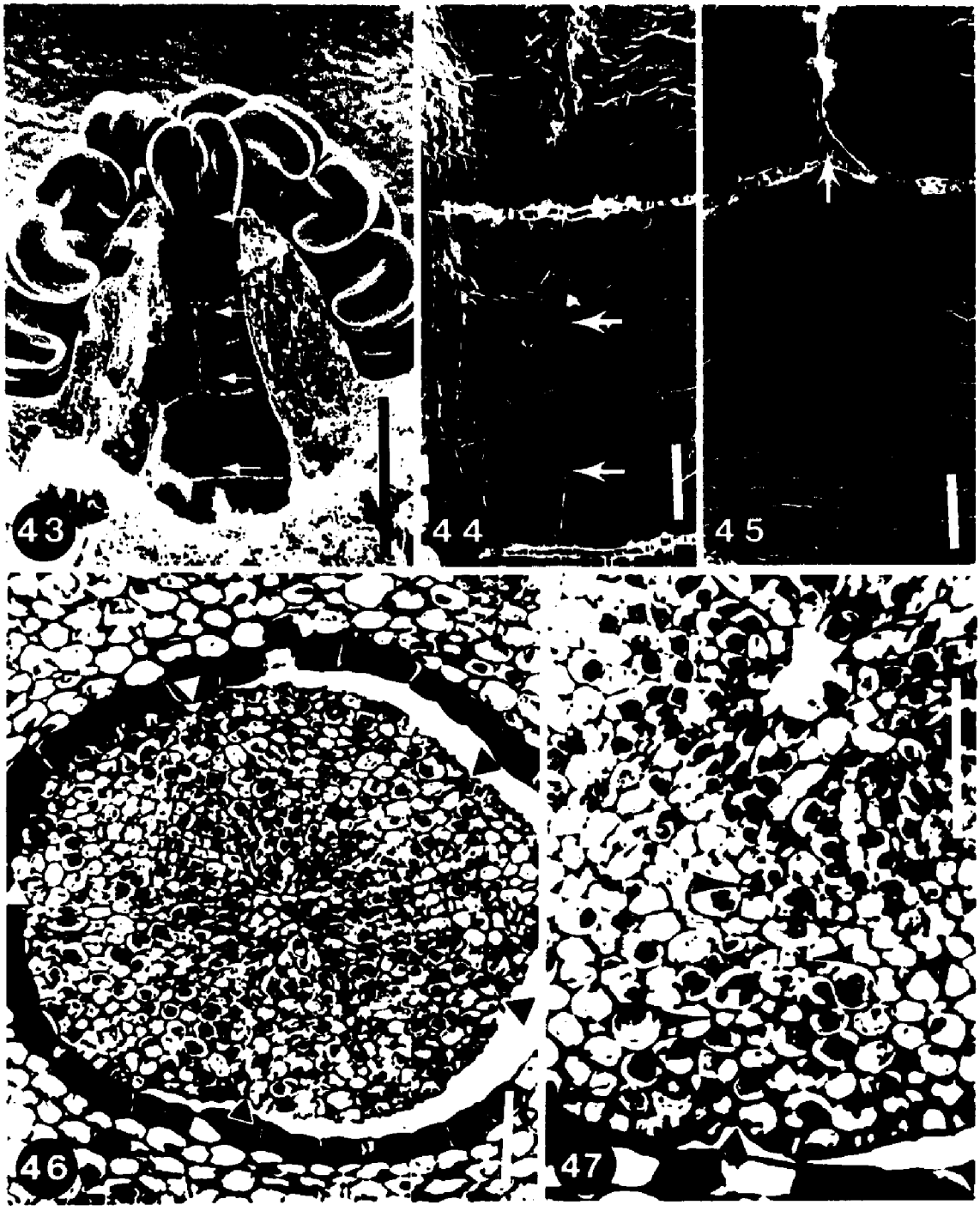
Figs. 43 - 47. Postgenital fusion of the style in H. acetosella. Scale bar = 500 uM for 43, Scale bars = 50 uM for 44 - 47. 43 - 45. Lateral view of flower bud with bracteoles, calyx, corolla, and facing half of androecial tube removed, showing paired stamens on the androecial tube, and the exsertion of the stylodes from the tube. The upper two arrows in 43 demarcate the area shown in 44, the lower two arrows in 43 the area shown in 45. The line of fusion between adjacent styles is only faintly visible in 44 (white arrows). The arrow in 45 indicates the basal termination of the zone of style fusion at the top of the ovary. Note there is no line of fusion visible in the connate ovary below the arrow. 46. Transverse section of the compound style of a flower bud that was at about the same stage of development as that in 43. Section is about mid-way between the style apex and the top of the locules. Triangles mark the five external sites of fusion of adjacent style members. 47. Transection from same flower as that in 46, showing periclinal divisions (pointers) in the epidermal layers of two adjacent style members after fusion. The triangle marks the external site of fusion.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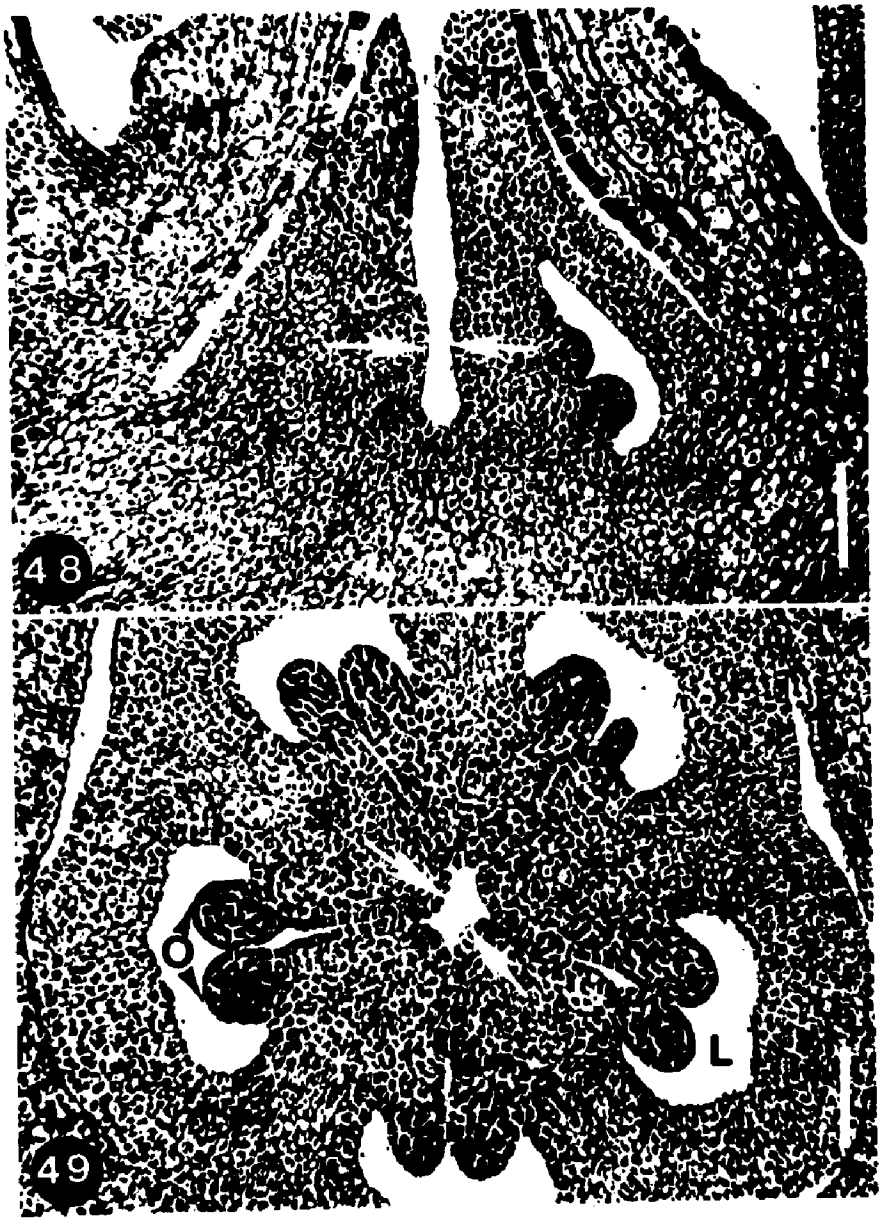
Figs. 48 and 49. Gynoecial anatomy in H. acetosella. Scale bars = 100 uM. 48. Longitudinal section, showing carpel development. The carpel on the right bears two ovule primordia, which are initiated from the base upwards. The floral meristem has been exhausted during carpel formation. 49. Transverse section of a gynoecium at a similar stage of development as that in 48. The five septa are formed from the congenital concrescence of the lateral carpel walls. Each locule bears two rows of ovules. The septa have become appressed due to lateral and centripetal growth. The white arrows in both figures point to the same advancing surfaces.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## CHAPTER THREE

# Floral Ontogeny in Hibiscus acetosella. II. Tissue Culture-Derived Teratomas


## ABSTRACT

Floral abnormalities were examined in tissue culture-derived plants of Hibiscus acetosella Welw. ex Hiern. Organs in mature flowers varied numerically, the number of stamens always less than in wild type, sepals, petals, carpels, and stylodes numbering both higher and lower than in wild type. Adjacent whorls of organs were correlated in their numerical variation. Ontogenetic examinations revealed abnormal development was manifested at all stages of development, including size of primordia, order of initiation within whorls, and relative location of primordia within and between whorls. Phylogenetic fusion of the style primordia and connation of the calyx and androecial tubes were frequently disturbed. Centripetal organ initiation, centrifugal androecium primordia initiation, and connation of the syncarpous ovary were the

only growth processes not affected. Petalody of the androecium occasionally occurred. Ovule formation on the androecial tube was common, and paralleled gynoecial ovule development. The androecial ovules were presumably functional as embryo sacs were present, even in some of the more aberrant ovules. It is possible that the floral abnormalities result from both genetic and epigenetic alterations that occurred during in vitro culture.

## INTRODUCTION

Teratology, or the study of abnormal structures, has long fascinated botanists. Teratology reached its zenith of popularity from the mid-1800s into the early-1900s. The most notable studies included Masters (1869), Penzig (1890), and Worsdell (1915, 1916). Such were the sentiments at that time concerning the use of teratology in elucidating the ontogeny and phylogeny of plant structures, that Masters (1869) described the controversy, "At one time it (teratology) was made to prove almost everything; what wonder that by some, now-a-days, it is held to prove nothing." The controversy continued. Lam (1961) was dubious as to the value of using teratomas in phylogenetic interpretations. Meeuse (1966) also regarded the use of teratomas in phylogenetic interpretations to be inappropriate, especially when the teratomas were caused by

metabolic irregularities or environmental influences. Conversely, other authors have cited teratological evidence in their interpretations of phylogenetic trends (Tepfer, 1953, Guedès, 1966). Reynolds and Tampion (1983) point out how teratological studies could provide botanists with information on organogenesis from a developmental viewpoint. Undoubtedly, the controversy regarding teratology will continue.

Meyer (1966) in the most recent review on floral abnormalities, points out how neglected this area of research has become, especially in view of currently available technology and knowledge. Though many floral abnormalities are heritable, Worsdell (1916) and earlier authors either did not have knowledge of or made no use of Mendelian genetics in their evaluations of teratomas. One application of teratology would be genetic evaluations of floral abnormalities, such as single gene, recessive inheritance of polycarpellate flowers in Lupinus angustifolius (Jaranowski, 1972), or single gene, dominant inheritance of androecial - borne ovules in Gossypium (Meyer and Buffet, 1962).

Scanning electron microscopy (SEM) with its much increased resolution has greatly enhanced examinations of floral ontogeny so that floral abnormalities can be studied at all stages of development, in the hopes of elucidating the patterns and temporal stages of development. In one recent study, male-sterility in two lines of Nicotiana

tabacum was shown to be respectively associated with cessation of stamen primordia growth and transformation of stamen primordia into petalodes (Rosenberg and Bonnett, 1983).

Reports on somaclonal variation, which is the variability generated by plant tissue culture, generally examine variation in agronomically useful traits such as yield or disease resistance (Evans and Sharp, 1986). There are few specific reports on variability of floral morphology. Syono and Furuya (1972) working with tobacco were the first to quantify differences in flower morphology in tissue culture-regenerated plants. Vajrabhaya (1977) described alterations in flower color, petal and sepal morphology in tissue culture-regenerated orchids. Reported herein is a study of the morphological variations in mature flowers from H. acetosella plants regenerated through tissue culture. Reported also is the ontogeny of the floral abnormalities, which to this author's knowledge is the first developmental examination of floral variability in tissue culture-regenerated plants. Variation in the vegetative morphology of these plants has been reported elsewhere (Ault, Reynolds, and Blackmon, 1986).

## MATERIALS AND METHODS

Wild type (WT) and tissue culture-derived (TC) plants were established in the greenhouse as previously described (Chapter One). Petal aestivation was examined in flower

buds the day before anthesis or the morning of anthesis as the petals began to unroll. The numbers of bracteoles, sepals, petals, stylodes, carpels, corolla width, and presence of ovules on the androecial tube were tabulated daily on open flowers of three WT and twenty-eight TC plants. The number of carpels was estimated by transversely cutting flowers with a razor blade and counting the number of ovule-bearing locules. The number of anthers per flower at anthesis was examined for the WT and fifteen of the TC plants by collecting ten flowers from each plant and counting the anthers under a dissecting microscope. Flower buds were collected from the TC plants for SEM. Ovules from the androecial tube and from carpels were collected prior to and on the day of anthesis for SEM and light microscopy (LM) studies. Processing of the materials for SEM and LM was the same as previously described in Chapter One. One-way analyses of variance (ANOVAs) were computed for the mean number of bracteoles, sepals, petals, carpels, stylodes, corolla widths, and anthers. Correlation coefficients were computed for all pairings of bracteole, sepal, petal, carpel, and stylode numbers for the TC plants. Dunnet's test for comparison of treatment means with a control mean was calculated for the number of anthers.

## TERMINOLOGY

The ontogenetic terminology is the same as in Chapter Two. The following additional terms have been used in this

chapter. Androecial ovules are ovules found on the androecial tube, in contrast with normal (gynoecial) ovules. Carpellody refers to the abnormal formation of a carpel or some portion thereof (style, ovule, etc.) in a nongynoecial position. Petalody is the the petal-like appearance of organs other than petals. Polyphylly is an increase in the number of members for any organ. Imbricate petal aestivation is any petal arrangement in bud in which the margins overlap. Convolute aestivation is one type of imbricate aestivation in which one petal margin sequentially overlaps the margin of the next petal; hence, the other petal margin is itself overlapped by the adjacent petal.

## RESULTS

Organography. The numbers of floral organs per flower at anthesis for wild type (WT) and tissue culture-derived (TC) plants of Hibiscus acetosella are shown in Table 1. Organ numbers were close to being uniform in the WT plants with seven to ten bracteoles, five to six petals and sepals (only one flower out of the 93 examined had six of each), five carpels, and five stylodes. Conversely, the number of flower organs for the TC plants varied considerably. Bracteoles ranged from zero to fifteen, sepals two to six, petals one to eight, carpels three to eight, and stylodes zero to ten. The differences among the plants varied significantly ($P < .001$) for the mean number of bracteoles, sepals, petals, carpels, and stylodes (Table 1). Mean

values of organs in the TC plants deviated both above and below those for the WT plants. Corolla widths of flowers with two or more petals also varied significantly ($P < .001$) among the plants (Table 1). Corolla width means for all of the TC plants were less than that for the WT plants.

Based on the assumption that "normal" flowers of H. acetosella bear seven to ten bracteoles and five each of all other floral organs, the tendency towards gain or loss of floral organs in the TC plants' flowers can be shown (Table 2). While both gains and losses were present for all organs, there was a greater tendency towards reduction in the number of bracteoles and towards proliferation in other organs. Of the 1576 flowers examined on TC plants, 511 exhibited either reduction or proliferation in the number of one or more of the organs sepals, petals, carpels, and stylodes.

Correlation coefficients were calculated to measure the degree of association among organ numbers in different whorls (Table 3). All combinations except bracteole-carpel number, bracteole-stylode number, and sepal-stylode number were highly significant ($P < .001$). Adjacent whorls showed a higher correlation; for example, the number of petals and sepals had a higher correlation ($r = .30$) than the number of petals and bracteoles ($r = .17$). The highest correlation was between the number of carpels and the number of stylodes ($r = .72$).

The number of anthers per flower at anthesis varied significantly ($P < .001$) among the plants (Table 4). WT

flowers averaged 65.3 anthers. The range for TC plants was 28.9 to 56.3 anthers. All of the TC plants had significantly fewer (P = .05) anthers than the WT.

The number of WT and TC flowers with convolute petal aestivation is shown in Table 5. Petals were convolute for 96% of the WT flowers with five petals and for 77% of the TC flowers with five petals, but only for 8% of the TC flowers with less or greater than five petals.

Organogenesis. It is not possible to describe the entire ontogeny of any given floral abnormality in the H. acetosella TC flowers, as the abnormalities were not uniform. That is, petal numbers in the TC plants ranged from one to eight instead of the normal five, and so forth. The developmental outcome of an aberration cannot be accurately predicted from a single observation. However, the SEM observations can be grouped into an ontogenetic sequence and the diversity of variations for a given stage of development, such as petal initiation, can then be described.

Aberrations were obvious at all stages of development in the H. acetosella TC flowers. Bracteole initiation was frequently incomplete around the circumference of the floral meristem, leaving gaps in the whorl (Figs. 1, 2). Bracteole primordia varied considerably in size (Fig. 5). Bracteoles either were initiated as separate primordia, or arose congenitally connate (Fig. 60). It is possible that some of

the larger primordia were the result of congenital connation of several primordia (Fig. 5). Bracteole primordia were frequently fewer in TC than WT flowers, which is in agreement with the data on mature flowers (Table 1).

The floral meristem circumscribed by bracteole initiation was often irregular in shape (Figs. 1 - 4). Sepal primordia varied in number and location (Figs. 4 - 10). The calyx tube frequently exhibited gaps at inception (Fig. 5) which persisted throughout development (Figs. 10, 12). When five sepals, the WT number, were initiated there was frequently variation in size (Fig. 9). Lateral and overarching growth of the sepals was variable, causing irregular (e.g. nonvalvate) aestivation (Figs. 8, 10, 11).

The petal-androecial ring meristems were generally visible prior to overarching growth of sepal primordia. The ring meristems were irregular in shape, the normal pentamerous configuration more the exception than the rule (Figs. 13 - 18). Petal primordia arose either slightly ahead of or at the same time as the primary androecial primordia (Figs. 14, 15). Petals were initiated either successively or simultaneously, and varied in number, distribution around the periphery, and lateral size (Figs. 15 - 24). As a result, primordia orientations were not strictly alternisepalous, as in the WT. The uneven size of the petal primordia persisted throughout development (Figs. 24, 26, 29). Due to both the different angles of insertion of the petal primordia and the anomalous shapes of the ring

primordia, the petals were not obliquely inserted relative to meristem radii, as in the WT. The pattern of petal aestivation frequently could not be predicted until the petal primordia had actually overlapped (Figs. 25 - 30). Most of the petals were free, but occasionally laterally connate petals were observed (Figs. 25, 26).

Androecial primordia development was also variable. The initial radial divisions in the ring primordia were irregularly placed, such that primary androecium primordia did not always arise in antipetalous positions (Figs. 14 - 16). The division of primary androecium primordia into secondary primordia, secondary into tertiary, and so forth, were all so irregular that the assignment of order number was not possible (Figs. 17 - 23). A variable number of stamens were then formed from any given primary primordia. Initiation of new androecium primordia was always centrifugal (Figs. 18 - 24). Division of the final order primordia into stamen-pairs still occurred (Figs. 24, 26), but the number of unpaired anthers increased. Abnormally shaped bisporangiate anthers were common, as were anthers composed of more than two sporangia (Fig. 31). Appendages, which appeared to be filaments arising from the filament tube without terminal sporangia, were common (Figs. 33, 43, 44). The filament tube was frequently not connate (Figs. 33, 34). Foliar-like extensions of the androecial tube were occasionally encountered (Figs. 32 - 34), which may form

the petaloid androecial appendages observed in several mature flowers (Figs. 62, 63).

The early carpel primordia were visible without removal of the androecial tube earlier in development than in the WT (Figs. 20, 21, 23). This was due to either earlier initiation of the carpel primordia, or to decreased growth of the androecial tube. The number of carpel primordia varied (Figs. 35 to 43). Carpel primordia orientation relative to the other organ whorls could not be determined as the organs in all whorls were unevenly distributed. The carpel primordia were generally equal in size, and appeared to be initiated simultaneously. However, smaller style branches were occasionally visible between the other primordia, as though some further primordia were centripetally initiated subsequent to the initial primordia (Fig. 41). The overarching growth and appression of the individual style primordia were irregular, even in flower primordia with five carpels (Figs. 36 to 39). The individual style branches frequently remained free or only partially fused (Figs. 42, 43). The compound ovary of the TC flowers was always connate, unlike the breaks in continuity frequently observed in both the calyx tube and the androecial filament tube.

Other floral abnormalities. Other abnormalities existed in select flowers in addition to the variations in the organs described above. Most of these were too infrequent to have

been observed in the ontogenetic examinations, and so are described only from flowers at anthesis. Two flowers were examined that had 15 bracteoles each in a distinct elongate helix on the receptacle. Some flowers exhibited adnation between bracteole and calyx, calyx and corolla, and androecium and gynoecium respectively. Several flowers were observed with petaloid structures connate to the androecial tube; one such bore a functional anther on the margin (Figs. 62, 63). Several flowers bore individual styles that arose from the distal end of the androecial tubes. Two flowers bore structures on the distal end of the androecial tubes that appeared to be small aberrant carpels, each with a single ovule. Both of these "carpels" were terminated by styles with stigmatic surfaces (Fig. 54). Petal color at anthesis also varied considerably, including yellow petals or yellow petals streaked with rose in addition to the normal rose-colored petals.

Androecial ovules. The number of flowers bearing androecial ovules at anthesis are shown in Table 6. None of the 94 WT flowers examined bore androecial ovules. Conversely, 22 of the 28 TC plants bore androecial ovules in one or more flowers. Androecial ovules were present in 10.5% of the TC flowers. The plant with the highest frequency of androecial ovules was TC 30 with 77.8%. The number of androecial ovules ranged from zero to six per flower.

The androecial ovules were always located at the distal end of the androecial tube, generally directly above or intermixed with the terminal anthers (Figs. 45 - 48, 61). The ovules arose either on the free stamen filaments, adjacent to the anthers (Fig. 51), or on terminal extensions of the filament tube. The earliest stage of androecial development observed was when the two integuments were enveloping the globular nucelli (Figs. 45 to 49). Gynoecial ovules isolated from the identical flower were of similar development (Fig. 50). Most of the androecial ovules examined at this stage were similar in appearance. Occasionally, aberrant forms were observed, such as one ovule with a single outer integument, two inner integuments, and two nucelli (Fig 48).

Aberrant forms are more evident in the androecial ovules more advanced in development. Most common is the incomplete development of the two integuments or the continued growth of the nucellus such that the nucellus was exposed at anthesis (Figs. 51 - 53). The ovules exhibited a variety of shapes, including reniform (the normal morphology), elongate, or irregularly lobed and twisted. Anatomical examinations revealed aberrations such as a double nucellus (Fig. 56), fused integuments, and a fused ovule and anther.

Comparison of normal-appearing androecial ovules and gynoecial ovules revealed their anatomy to be similar, both crassinucellate, amphitropous ovules with two integuments

and curved embryo sacs (Figs. 55, 57). Both types of ovules were supplied by single vascular strands which traversed the funiculus of the gynoecial ovule and the "filament" of the androecial ovule. Presumptive functional embryo sacs were observed in the normal-appearing androecial ovules (Figs. 58, 59). Embryo sacs were occasionally observed in the aberrant androecial ovules, including one with two functional-appearing embryo sacs. The fertility of the androecial ovules was not determined as they dehisced along with the corolla and androecial tube two or three days after flower anthesis.

## DISCUSSION

The flowers of the tissue culture-derived H. acetosella plants were highly variable in organ numbers. Of the 1576 flowers examined from the TC plants, 32% varied in number in one or more of the floral organs. Polyphylly, or a proliferation of organs, occurred more frequently than a loss of parts. Polyphylly is a common teratological phenomenon (Guedès, 1966). The multiplication of organs has been ascribed to either partitioning of primordia or increase in the number of whorls (Guedès, 1966). There is evidence for both in H. acetosella. In young material, petal primordia were frequently observed that were adjacent and individually smaller than the other petal primordia, as if a primordium had split, forming two petals. Stylodes were occasionally observed to bear a second, smaller stylode

branching from the former above the point of the divergence of all the stylodes of the compound style. The branching of the stylodes to form secondary stylodes would in part explain the observed discrepancy in the stylode to locule ratio, which should be unity in this species. Whorl number increase was rare. Two flowers were observed each with 15 bracteoles arranged in an elongate helix from the pedicel to the base of the calyx tube. Carpel primordia were observed centripetal to the initial gynoecial whorl.

Floral organs may be lost due to a lack of induction of the primordia, or may appear to be lost when primordia development is suppressed at very early stages, such that no discernible trace is apparent in the mature flower (Tucker, 1984a). Organ reduction within the TC flowers apparently reflects lack of initiation. No ontogenetic evidence to explain missing sepals, petals, or carpels as having been initiated and subsequently suppressed was found. Although specimens were not sectioned to document that primordia initiation in terms of initial cell divisions had not occurred, Tucker (1984b) knew of no examples in which an organ was initiated and a protuberance failed to become visible.

Petaloid stamens and ovules on the androecial tubes provided evidence of morphological or functional transformation of one organ type into another. Similar transformations in other teratomas have been cited as evidence for homology of floral organs. Tepfer (1953) cited

teratological flowers of Aquilegia in which carpellate-appearing stamens with submarginal pollen sacs were evidence for homology of stamens and carpels. Guedès (1966) likewise cited several teratological examples of intermediate floral structures as evidence for homology of stamens and carpels. However, criticism of teratological evidence has been raised numerous times (Masters, 1866, Lam, 1961, Meeuse, 1966); therefore phylogenetic interpretations of teratomas must be taken with caution.

Petal aestivation in the abnormal flowers was predominantly associated with petal number, as 77% of the flowers possessing five petals exhibited convolute aestivation; whereas, 8% of the flowers with less or greater than five petals were convolute. Tucker (1984a) stated that petal aestivation in leguminous flowers appears to result from changes in petal shape at mid-developmental stages. It was demonstrated that petal aestivation in WT H. acetosella is determined very early in the development of the petal primordia, due to the uneven division of primary androecial primordia, oblique insertion of petal primordia at inception, and the eventual canting of the petal margins (Chapter Two). The formation of an abnormal number of petal primordia in the TC plants changes the relative orientation of the primordia. Petal aestivation becomes a random event, determined during the lateral extension of adjacent petals and influenced by the individual petal primordia size, shape, and orientation. This was also true for many of the

TC flowers with five petals, judging by the decrease in percentages of convolute petal aestivation.

The style primordia in WT H. acetosella have been demonstrated to postgenitally fuse (Chapter Two). The style primordia frequently were not fused, or only partly fused in the TC flowers. This could result from a) lack of correlated growth between primordia, such that style surfaces were not brought into contact, b) absence of sufficient mechanical pressure by the androecial tube (and perhaps also by the calyx and corolla) on the developing style primordia such that again the primordia surfaces were not brought together, or c) loss of the ability by the style protodermal cells to divide periclinally or to deposit additional cell wall matrix-materials such that fusion would not occur even if the primordia were appressed. The aberrant carpel primordial growth and the poor development of the androecium appears to support the first two hypotheses. The last hypothesis would have to be examined cytologically, which is beyond the scope of the current study.

Cusick (1956), interpreting the results of organogenesis in bisected flower primordia of successive stages of development, hypothesized that "...a developing flower passes through a succession of physiological states that permit and regulate the formation of each kind of organ in turn." Wardlaw (1957) refers to the floral meristem as a reaction center which passes through an irreversible series

of phases. In response to some environmental cue, an eliciting substance moves into the apical reaction center (meristem). The action of a specific set of genes is induced, followed by a specific response such as the initiation of the first whorl of floral organs. The physiological changes in turn lead to a new set of genes being triggered, and so on, until the floral meristem has been exhausted or all organs formed. Heslop-Harrison (1964) hypothesized that the organs of a flower are initiated in response to the function of a relay system of gene activation. Conceptually the gene complexes would be promoted by a series of specific inducers which diffuse through the floral meristem from the prior site of organ initiation to the next site of induction. Heslop-Harrison also hypothesized that in addition to the short-range control system (e.g. the diffusive inducers) there is also a generalized control mechanism mediated through the hormone (or some other mediator) metabolism of the whole plant. Both Wardlaw and Heslop-Harrison theorized how teratological phenomena could be interpreted in light of their theories. Mutations in any of the gene complexes of the reaction center (meristem) could lead to phenotypic variation in the resultant organ. Mutation or variation within the whole plant control mechanism could result in the continued expression of one gene complex, resulting in such phenomena as "double flowers" which arise from the continued production of petals (Heslop-Harrison, 1964). If a mutant

gene is expressed in one of the early gene complexes of the reaction center, the more likely it will be that all subsequent phases of development will be affected.

Though floral organs were frequently reduced in number, there was never the total absence of an organ whorl. This again supports Heslop-Harrison and Wardlaw in that formation of organ primordia is the reflection of an influence emanating from existing organs. However, Hicks and Sussex (1971) presented evidence in which organ primordia inception was not necessarily dependent on the existence of older primordia. Therefore they felt that progressive influences more likely emanated from the flower center rather than along an acropetal gradient from organ whorl to whorl.

Androecial development was greatly altered as the TC flowers bore significantly fewer stamens, pairing of the stamens was less frequent, and the stamens could no longer be visualized in whorls vertically or rows laterally as in WT. The normal sequence of secondary through quaternary androecial primordia formation could not be detected in the TC flowers, though various androecial primordia divisions still occurred prior to stamen differentiation. These aberrations may be due to the nature of stamen formation. Other floral organs are initiated directly from the floral meristem; whereas, stamens normally develop after a series of intervening primordia-divisions. Under Heslop-Harrison's theory of control of floral development by sequential gene complexes, it seems possible that the more involved the

developmental pathway, the more genes likely involved in regulation of the development. If this is valid, this allows more "opportunities" for genetic or epigenetic alterations to occur, resulting in a greater number of manifested aberrations.

The complex development of the malvaceous androecium has been cited as evidence for different theories on androecial phylogeny. Saunders (1936) and Rao (1952) regarded the malvaceous androecium to have arisen from the "dedoublement" of a single whorl of stamens, the inner whorl having since disappeared. Van Heel (1966, 1969) felt the pairing of bithecous stamens was evidence for the phylogenetic splitting of a tetrasporangiate stamen into two "half-stamens", or that the stamens of the Malvaceae are a derived condition. Both Wilson (1937) and Melville (1963) felt the bisporangiate stamen in the Malvaceae was a primitive, not a derived condition. Wilson interpreted the complex androecial development as evidence for the derivation from a dichotomouly-branched system of fertile and sterile telomes. It is difficult to reconcile the general trend towards reduction and fusion in the androecium, as cited by all of these authors, with a concurrent superficial splitting of the individual stamens, as proposed by van Heel; it therefore seems more likely that the evidence supports Wilson and Melville in that the bisporangiate stamen in the Malvaceae is a basic condition in which the penultimate dichotomies have failed to unite.

The teratological data presented here, as far as such information can be trusted in phylogenetic interpretations, tends to lend support to Wilson and Melville. If teratomas tend to revert to an ancestral state, then tetrasporangiate stamens should have been observed according to van Heel's interpretation of the malvaceous stamen; however, tetrasporangiate stamens were never observed in the teratomas.

Most of the organs in the TC plants were correlated in their numerical variations. Syono and Furuya (1972) observed a similar correlation of calyx, corolla, and stamen number in variant tissue culture tobacco plants. Ellstrand (1983) reported a correlation of all organs except the gynoecium in numerically variable natural populations of Ipomopsis aggregata. This supports Heslop-Harrison's theory of two (or possibly more) levels of control over organogenesis. The discrete gene complexes controlling organ location, development, etc., could be altered, but a presumably overriding genetic control coding for correlation of organ number is still in effect.

Other developmental sequences in the TC flowers were also not altered. Centripetal initiation of the different organ whorls and centrifugal primordia initiation within androecia were not affected. Flowers were not observed with breaks in the congenitally connate ovary bases, though breaks in the continuity of the calyx and androecial tubes were observed. It appears that certain fundamental

developmental patterns were not altered, or could not be altered without disrupting the ability to flower, again in agreement with Heslop-Harrison's theory of floral organogenesis being controlled at several levels.

Genetic abnormalities are common in tissue culture regenerated plants, including changes in ploidy, gene mutations, deletions, rearrangements, and amplifications (Larkin and Scowcroft, 1981, Reisch, 1983, Evans and Sharp, 1986). Genetic alterations in the TC H. acetosella plants have also likely occurred, as evidenced by the reduced fertility in most plants and the changes in ploidy in select individuals (see Chapter One). It is not known which, if any, of the floral abnormalities are genetic in nature, as the few progeny of the tissue culture plants that have bloomed to date have reverted back to wild type in terms of floral morphology. Most of the tissue culture plants were self-incompatible (Chapter One), which rules out elucidating by progeny testing whether or not the floral variations were genetic in these individuals. The floral variations have occurred through two blooming cycles of the tissue culture plants, which could indicate genetic alterations. However, epigenetic changes, or changes in gene expression, are common in tissue culture plants, and can persist for at least two years after removing the plants from the tissue culture environment (Hussey, 1983).

Meyer (1966) lists four causal agents of floral abnormalities: unusual climatic conditions, heritable

(genetic) abnormalities, parasitism, and chemical treatment. She cites numerous examples of each. The possibility of genetic abnormalities is likely, as discussed above. It is possible that the chemicals employed in the tissue culture system induced either (or both) genetic and epigenetic alterations in the regenerated plants, as the chemical composition of culture media has been reported to enhance plant variability (Evans and Sharp, 1986). The wild type plants that were grown as controls along with the tissue culture plants never exhibited any floral abnormalities, which would appear to rule out any climatic or parasitic influences. However, these influences could have an effect on the tissue culture plants if their genomes were altered, as the phenotypic expression of a plant results from "interaction of its genes and cytoplasm with each other and with its internal (physiological) and external environment" (Meyer, 1966).

There appears to be a general correlation between the presence of floral abnormalities, reduced fertility (Chapter One), and vegetative abnormalities (Ault, et al, 1986) as all three were generally present in the TC plants. However, there were exceptions: TC 11 was self-infertile, but bore all normal flowers (Table 1). The presence of androecial development-abnormalities probably contributed to plant infertility, as pollen development was also likely affected. Pollen morphology was not examined, and pollen viability was tested only via self-fertilizations (Chapter One). Genetic

or epigenetic variations accumulated during the tissue culture phase would presumably have an effect on both fertility and morphology (vegetative and floral). It seems unlikely that mutations would accumulate affecting only one of these traits while no mutations arose affecting the others.

Botanists regard reports on teratological plant materials with considerable trepidation. This bias against information that does not fit into "normal" or "acceptable" hypothetical frameworks may in part be because a) teratological information has frequently in the past been reported in a scientifically unsound manner, b) many teratomas are inherently unstable or unrepeatable, and so difficult to examine, and c) naturally occurring teratomas may be quite rare and so difficult to sufficiently collect for evaluation. In light of these points, it is interesting to note the considerable interest in somaclonal variation. It is this author's contention that evaluations of variant tissue culture-derived plants is no more than teratology couched under a different name. Semantics aside, evaluations of plant abnormalities have a legitimate place in botany. Plant tissue culture can yield large numbers of heritable or stabile plant variations that can be used in as diverse of activites as introducing useful mutations into crops to elucidating molecular controls of plant development. It is hoped that the combination of a ready technique of generating variant plants coupled with current

research capabilities brings favor back to the wrongfully neglected area of teratology.

Androecial ovules. The formation of carpels or ovules in abnormal positions (carpellody or pistillody) has been frequently reported. Marx and Mishanec (1964) reported the formation of rudimentary carpels on foliage leaves of Pisum. Lutz (1897) and Worsdell (1916) reported tulips and Crocus bearing stigmas, carpels, or ovules on bracts, perianth members, and stamens. Carpellody of the androecium is the most frequent transformation. Meyer (1966) lists 65 genera in which carpellody of the androecium has been reported. Carpellody of the androecium in the Malvaceae has been reported in Gossypium (Meyer and Buffet, 1962).

Carpellody of the androecium has been reported to be heritable in some plants. Masters (1869) discussed the recessive heritability of stamens transformed into carpels in Cheiranthus. Meyer and Buffet (1962) reported the androecial ovule inheritance in a Gossypium hybrid to be from a dominant gene. The heritability of androecial ovules in H. acetosella has not been fully investigated. The tissue culture clone with the greatest expression of androecial ovules, TC 30, proved to be self-sterile (see Table 1, Chapter One), as was true for many of the other clones that bore androecial ovules. One clone that bore androecial ovules in high numbers and was self - fertile was TC 4. No androecial ovules have been observed in the

progeny of this plant as they have come into bloom. Heritability of the androecial ovules cannot be ruled out at least until outcrosses are attempted between several of the ovule-bearing TC clones. Even if the presence of androecial ovules proves to be nonheritable, the TC clones bearing ovules can be maintained indefinitely by cuttings. The expression of the androecial ovules appears to be stable over time, as their presence has been observed over two blooming cycles in several of the TC plants.

Androecial ovules have occasionally been reported to be functional and capable of forming seed after fertilization. Masters (1869) described fertile ovules in the stamen whorls of Papaver somniferum and Cheiranthus cheiri. The potential for fertility in H. acetosella androecial ovules in situ cannot be evaluated due to the ephemeral nature of the androecium after anthesis. Pollination of the androecial ovules in vitro may be possible with isolated floral buds or ovules, as in vitro pollination or ovule culture has been successful with a variety of plants (Murashige, 1978).

The anatomical observations of the androecial ovules indicate that the potential for fertility exists, as presumptive functional embryo sacs are present. It is interesting that embryo sacs were present even in some of the more aberrant ovules. The presence of functional embryo sacs in otherwise aberrant carpellary ovules has been reported in Gossypium ((Joshi, Wadhwani, and Johri, 1967).

It appears that the development of the more normal-appearing androecial ovules parallels both the development and time course of the gynoecial ovules. This presents the opportunity for physiological and developmental observations of ovule formation in situ, without the damage and resultant disturbance brought about by removal of a portion of the carpel wall. The androecial ovules can be exposed at early stages of development by simply prying open the calyx and corolla.

The androecial ovules were present only at the apex of the androecial tube and invariably occurred above the uppermost anthers. The androecial ovules reported in Gossypium were also apical on the androecial tube (Meyer and Buffet, 1962). There is at present no good explanation as to the specific location of the androecial ovules. It is interesting to note that as the anthers of the Malvaceae are centrifugal, the androecial ovules are presumably arising adjacent to already well-developed organs, and are forming on tissue (the filament tube) that otherwise would not differentiate into another organ type.

The morphology and anatomy of the aberrant androecial ovules bears a marked resemblance to the "motes" or aberrant gynoecial ovules of cotton (after accounting for the basic difference of anatropy and a straight embryo sac in cotton), as described by Joshi, Wadhwani, and Johri (1967). The aforementioned authors ascribed the development of motes in cotton to insect damage, unusual climatic conditions, and

defective nutrition to the developing ovules. As previously discussed, the first two factors seem unlikely to have influenced the TC plants. "Defective nutrition" causing aberrant development of the androecial ovules also appears unlikely. The vascular connections to the androecial ovules appeared to be as well-developed as in the gynoecial ovules. The rapidity of the development of the androecial ovules (after differentiation of all the anthers) indicates the androecial ovules were acting as effective nutrient sinks. Instead aberrant development may have been in part due to the abnormal location of the androecial ovules away from the physiological and mechanical influences the gynoecium undoubtedly has on normal ovule development. It is also likely that the genetic and epigenetic mechanisms underlying the abnormal TC floral developments also affected the androecial ovules. The gynoecial ovules in the TC flowers may also have exhibited aberrations which were not examined.

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Table 1. Numbers of floral organs and corolla width at anthesis for wild type and tissue culture-derived H. acetosella plants.

| Plant[a] | Bracteoles | Sepals | Petals | Carpels[b] | Stylodes[c] | Widths (cm)[d] |
|---|---|---|---|---|---|---|
| WT | 9.2 ± 0.1 (93)[e] | 5.0 ± 0.0 (93) | 5.0 ± 0.0 (93) | 5.0 ± 0.0 (78) | 5.0 ± 0.0 (93) | 7.5 ± 0.1 (88) |
| TC 2 | 9.4 ± 0.1 (59) | 5.0 ± 0.0 (59) | 5.0 ± 0.1 (59) | 5.0 ± 0.1 (23) | 5.1 ± 0.1 (63) | 6.5 ± 0.1 (58) |
| TC 3 | 9.1 ± 0.1 (52) | 5.0 ± 0.0 (52) | 5.0 ± 0.0 (53) | 5.0 ± 0.0 (23) | 5.0 ± 0.0 (54) | 4.2 ± 0.1 (51) |
| TC 4 | 8.0 ± 0.2 (52) | 5.0 ± 0.1 (55) | 5.0 ± 0.1 (55) | 5.2 ± 0.1 (44) | 5.4 ± 0.1 (56) | 5.9 ± 0.2 (52) |
| TC 5 | 7.9 ± 0.3 (51) | 4.9 ± 0.1 (52) | 4.9 ± 0.1 (51) | 5.0 ± 0.1 (33) | 5.3 ± 0.1 (54) | 6.3 ± 0.2 (46) |
| TC 6 | 8.3 ± 0.3 (29) | 5.1 ± 0.1 (32) | 4.9 ± 0.2 (33) | 5.0 ± 0.1 (21) | 5.1 ± 0.1 (34) | 6.0 ± 0.3 (28) |
| TC 7 | 9.1 ± 0.2 (54) | 5.1 ± 0.0 (54) | 5.1 ± 0.0 (54) | 5.1 ± 0.0 (44) | 5.1 ± 0.0 (56) | 4.1 ± 0.1 (54) |
| TC 8 | 7.8 ± 0.3 (56) | 5.2 ± 0.1 (56) | 4.9 ± 0.1 (56) | 5.1 ± 0.1 (37) | 5.1 ± 0.1 (51) | 6.3 ± 0.2 (54) |
| TC 9 | 9.2 ± 0.1 (50) | 5.1 ± 0.0 (50) | 5.1 ± 0.1 (50) | 5.0 ± 0.0 (36) | 5.1 ± 0.0 (73) | 4.3 ± 0.1 (50) |
| TC 10 | 8.3 ± 0.2 (62) | 4.9 ± 0.0 (62) | 4.9 ± 0.1 (69) | 5.1 ± 0.1 (49) | 5.1 ± 0.1 (71) | 6.3 ± 0.1 (60) |
| TC 11 | 8.6 ± 0.1 (57) | 5.0 ± 0.0 (57) | 5.0 ± 0.0 (62) | 5.1 ± 0.0 (44) | 5.0 ± 0.0 (50) | 6.7 ± 0.1 (57) |
| TC 12 | 9.1 ± 0.1 (50) | 5.0 ± 0.1 (50) | 5.0 ± 0.0 (50) | 5.1 ± 0.0 (23) | 5.1 ± 0.0 (54) | 3.8 ± 0.1 (46) |
| TC 13 | 9.3 ± 0.1 (54) | 5.1 ± 0.0 (54) | 5.1 ± 0.0 (54) | 5.0 ± 0.0 (23) | 4.9 ± 0.0 (52) | 4.8 ± 0.1 (54) |
| TC 16 | 8.5 ± 0.4 (10) | 5.3 ± 0.2 (10) | 5.4 ± 0.2 (10) | 5.0 ± 0.0 (2) | 5.2 ± 0.1 (10) | 3.5 ± 0.1 (10) |
| TC 17 | 6.4 ± 0.2 (51) | 4.7 ± 0.1 (51) | 4.6 ± 0.1 (51) | 5.0 ± 0.1 (50) | 4.9 ± 0.1 (52) | 5.4 ± 0.1 (50) |
| TC 18 | 8.1 ± 0.2 (16) | 4.9 ± 0.1 (16) | 5.3 ± 0.2 (16) | 5.5 ± 0.2 (8) | 5.9 ± 0.3 (16) | 4.8 ± 0.2 (15) |
| TC 19 | 9.1 ± 0.1 (58) | 5.1 ± 0.0 (58) | 5.1 ± 0.0 (58) | 5.2 ± 0.1 (37) | 5.2 ± 0.0 (59) | 5.5 ± 0.1 (53) |
| TC 20 | 7.2 ± 0.3 (72) | 5.2 ± 0.1 (72) | 5.1 ± 0.1 (72) | 5.3 ± 0.1 (18) | 5.4 ± 0.1 (72) | 4.6 ± 0.1 (71) |
| TC 21 | 8.4 ± 0.2 (51) | 5.0 ± 0.0 (51) | 5.0 ± 0.0 (51) | 5.1 ± 0.0 (30) | 5.0 ± 0.0 (51) | 5.4 ± 0.1 (50) |
| TC 22 | 7.5 ± 0.4 (11) | 5.4 ± 0.2 (11) | 5.3 ± 0.2 (11) | 5.0 ± 0.0 (3) | 5.6 ± 0.2 (11) | 4.6 ± 0.3 (11) |
| TC 23 | 8.8 ± 0.2 (41) | 5.0 ± 0.0 (41) | 5.0 ± 0.1 (41) | 5.0 ± 0.0 (4) | 5.2 ± 0.1 (41) | 5.6 ± 0.1 (40) |
| TC 24 | 8.3 ± 0.2 (56) | 5.0 ± 0.1 (56) | 5.3 ± 0.1 (56) | 5.5 ± 0.1 (37) | 5.6 ± 0.1 (58) | 4.6 ± 0.1 (55) |
| TC 25 | 7.4 ± 0.3 (40) | 4.9 ± 0.1 (40) | 5.3 ± 0.1 (40) | 5.4 ± 0.1 (20) | 5.5 ± 0.1 (40) | 4.9 ± 0.2 (35) |
| TC 26 | 8.6 ± 0.3 (28) | 5.1 ± 0.1 (28) | 5.1 ± 0.2 (28) | 5.0 ± 0.0 (4) | 5.2 ± 0.1 (28) | 5.3 ± 0.2 (28) |
| TC 27 | 6.6 ± 0.2 (61) | 4.7 ± 0.1 (61) | 4.8 ± 0.1 (61) | 4.9 ± 0.1 (64) | 5.0 ± 0.1 (61) | 6.1 ± 0.1 (60) |
| TC 28 | 8.5 ± 0.1 (35) | 5.0 ± 0.0 (35) | 5.0 ± 0.0 (35) | 5.0 ± 0.0 (19) | 5.1 ± 0.0 (35) | 5.0 ± 0.2 (31) |
| TC 29 | 8.7 ± 0.1 (40) | 5.4 ± 0.1 (40) | 5.2 ± 0.1 (40) | 5.1 ± 0.1 (34) | 5.1 ± 0.1 (40) | 4.9 ± 0.2 (37) |
| TC 30 | 8.8 ± 0.1 (65) | 5.2 ± 0.1 (65) | 5.2 ± 0.1 (65) | 5.1 ± 0.1 (32) | 5.0 ± 0.1 (65) | 4.5 ± 0.1 (65) |
| TC 31 | 9.6 ± 0.1 (14) | 5.2 ± 0.1 (14) | 5.3 ± 0.2 (14) | 5.3 ± 0.2 (9) | 5.3 ± 0.1 (14) | 5.1 ± 0.2 (14) |
| RANGE[f] | | | | | | |
| WT | 7 - 10 | 5 - 6 | 5 - 6 | 5 | 5 | 5.0 - 8.9 |
| TC | 0 - 15 | 2 - 6 | 1 - 8 | 3 - 8 | 0 - 10 | 2.2 - 9.0 |
| ANOVA[g] | F = 16.52 | F = 5.34 | F = 2.46 | F = 3.45 | F = 6.14 | F = 49.89 |
| | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |

[a] WT = wild type, pooled data from three plants. TC = tissue culture.
[b] Carpel number inferred from number of locules per ovary.
[c] Number of style branches arising from compound styles.
[d] Width measurements from flowers with two or more petals.
[e] Values are means ± standard errors. Sample sizes are in parenthesis.
[f] Range values are for all flowers from the wildtype (WT) and tissue culture (TC) plants.
[g] F values and significance levels (P) are from one-way ANOVAs.

Table 2. The number of flowers from tissue-culture plants of H. acetosella with fewer or more organs than wild type.

| Organs[a] | Fewer | More |
|---|---|---|
| Bracteoles | 123 | 29 |
| Sepals | 87 | 141 |
| Petals | 116 | 185 |
| Carpels | 27 | 100 |
| Stylodes | 40 | 243 |

[a] The number of floral organs in the WT are assumed to be 7 to 10 bracteoles and 5 each of all other organs. This is based on collected data and taxonomic descriptions.

Table 3. Correlation coefficients (r-values) among flower organ numbers in tissue culture-derived plants of H. acetosella.

| | Bracteoles | Sepals | Petals | Carpels[a] | Stylodes[b] |
|---|---|---|---|---|---|
| Bracteoles | -- | .18*** | .17*** | .00NS | -.02NS |
| Sepals | | -- | .30*** | .15*** | .06NS |
| Petals | | | -- | .22*** | .18*** |
| Carpels | | | | -- | .72*** |

*** P < .001, NS = not significant at P = .05.
[a] Carpel number inferred from number of locules in compound ovary.
[b] Stylode = style branches arising from compound style.

Table 4. Number of anthers per flower for wild type (WT) and tissue-culture-derived (TC) plants of H. acetosella.

| Plant | Anthers[a] |
|---|---|
| WT | 65.3 ± 1.4 |
| TC 3 | 40.4 ± 1.1 |
| TC 6 | 45.8 ± 0.9 |
| TC 7 | 43.9 ± 1.0 |
| TC 11 | 52.2 ± 1.7 |
| TC 12 | 43.4 ± 0.9 |
| TC 15 | 28.9 ± 1.3 |
| TC 16 | 40.1 ± 1.6 |
| TC 17 | 56.3 ± 1.5 |
| TC 19 | 43.0 ± 2.4 |
| TC 20 | 34.4 ± 2.7 |
| TC 22 | 44.6 ± 1.7 |
| TC 24 | 37.4 ± 1.3 |
| TC 25 | 49.0 ± 1.8 |
| TC 30 | 42.2 ± 0.9 |
| TC 31 | 38.8 ± 2.1 |
| | $F = 29.24$[b] |
| | $P < 0.001$ |

[a] Means ± SE calculated from 10 flowers per plant.

[b] F-statistics based on one-way ANOVA. A difference of more than 4.71 anthers between WT and each TC value is significant (P = .05) according to Dunnet's test for comparison of a control mean with treatment means.

Table 5. Variation in petal aestivation in wild type (WT) and tissue-culture-derived (TC) plants of H. acetosella.

| | 5 Petals | | ≠ 5 Petals | |
|---|---|---|---|---|
| Clone | Convolute | Various[a] | Convolute | Various |
| TC 2 | 39 | 13 | 0 | 12 |
| TC 3 | 40 | 12 | 1 | 2 |
| TC 4 | 7 | 6 | 0 | 8 |
| TC 5 | 8 | 5 | 0 | 4 |
| TC 6 | 14 | 6 | 0 | 2 |
| TC 7 | 28 | 3 | 0 | 2 |
| TC 8 | 28 | 8 | 0 | 5 |
| TC 9 | 12 | 2 | 0 | 2 |
| TC 10 | 25 | 6 | 0 | 4 |
| TC 11 | 50 | 2 | 0 | 2 |
| TC 12 | 26 | 0 | 0 | 0 |
| TC 13 | 6 | 3 | 0 | 2 |
| TC 16 | 1 | 1 | 0 | 1 |
| TC 17 | 39 | 22 | 1 | 9 |
| TC 18 | 9 | 1 | 0 | 0 |
| TC 19 | 64 | 3 | 1 | 1 |
| TC 20 | 14 | 8 | 1 | 6 |
| TC 21 | 9 | 0 | 0 | 1 |
| TC 22 | 10 | 9 | 1 | 6 |
| TC 23 | 5 | 2 | 1 | 1 |
| TC 24 | 5 | 12 | 0 | 10 |
| TC 25 | 17 | 14 | 1 | 3 |
| TC 26 | 6 | 3 | 0 | 0 |
| TC 27 | 5 | 7 | 0 | 3 |
| TC 28 | 7 | 0 | 0 | 0 |
| TC 29 | 16 | 2 | 1 | 3 |
| TC 30 | 23 | 5 | 0 | 0 |
| TC 31 | 18 | 2 | 0 | 3 |
| TOTAL | | | | |
| ALL TC | 531 | 157 | 8 | 92 |
| WT | 48 | 2 | 0 | 1 |

[a] Imbricate (overlapping), but nonconvolute.

Table 6. Ovule production on androecial tubes of H. acetosella.

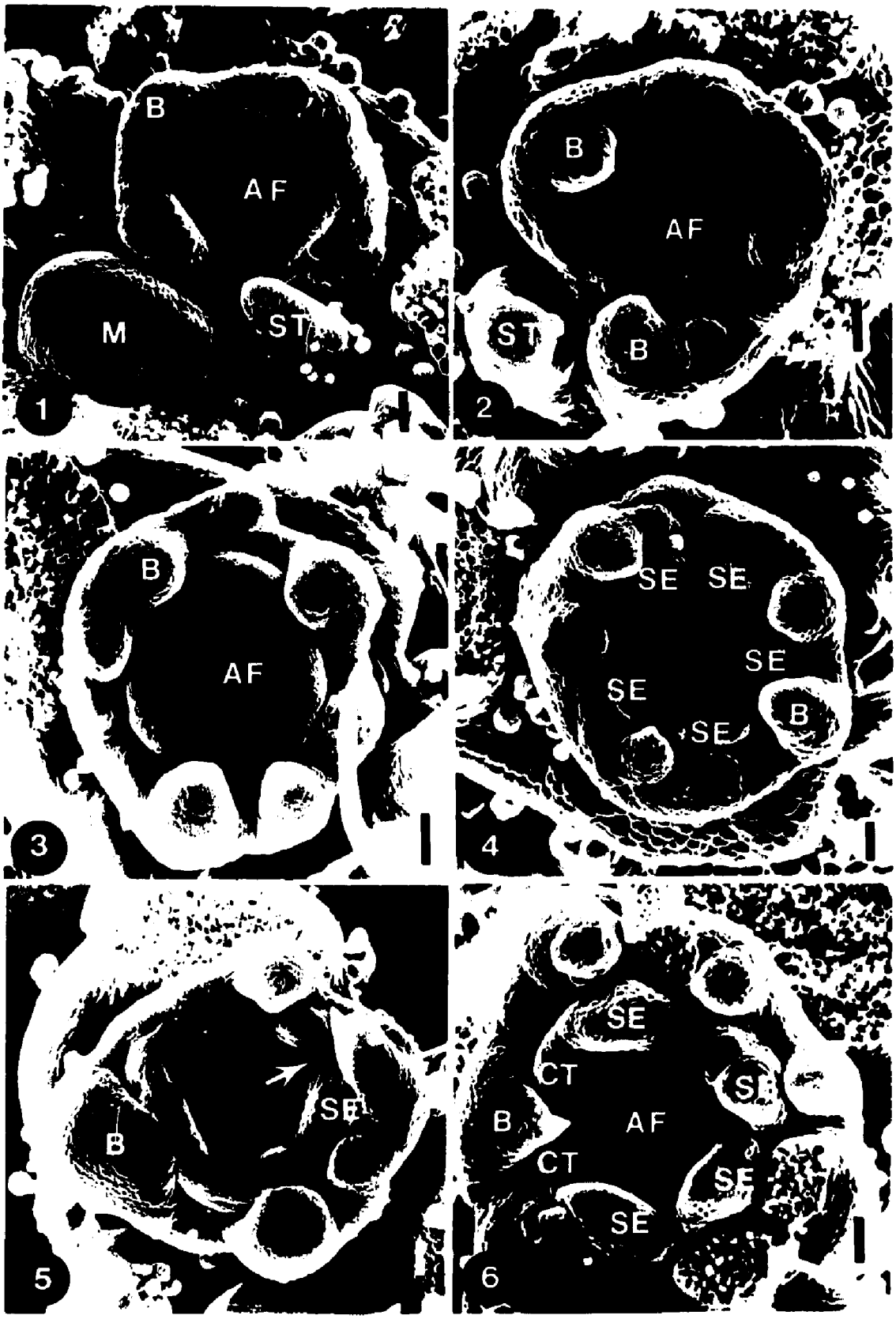
| Plants[a] | Androecial[b] ovules (%) | Flowers examined (No.) |
|---|---|---|
| WT | 0.0 | 94 |
| TC 2 | 1.7 | 59 |
| TC 3 | 7.4 | 54 |
| TC 4 | 23.6 | 55 |
| TC 5 | 11.3 | 53 |
| TC 6 | 11.8 | 34 |
| TC 7 | 3.7 | 54 |
| TC 8 | 8.9 | 56 |
| TC 9 | 12.0 | 50 |
| TC 10 | 9.9 | 71 |
| TC 11 | 1.4 | 71 |
| TC 12 | 4.0 | 50 |
| TC 13 | 5.6 | 54 |
| TC 16 | 0.0 | 10 |
| TC 17 | 8.0 | 50 |
| TC 18 | 18.8 | 16 |
| TC 19 | 0.0 | 58 |
| TC 20 | 12.5 | 72 |
| TC 21 | 0.0 | 51 |
| TC 22 | 27.2 | 11 |
| TC 23 | 7.3 | 41 |
| TC 24 | 10.7 | 56 |
| TC 25 | 2.5 | 40 |
| TC 26 | 0.0 | 28 |
| TC 27 | 6.6 | 61 |
| TC 28 | 0.0 | 35 |
| TC 29 | 0.0 | 40 |
| TC 30 | 77.8 | 63 |
| TC 31 | 7.1 | 14 |
| All TC | 10.5 | 1307 |

[a] WT = wild type. TC = tissue-culture-derived.

[b] Percentages based on presence or absence of ovules on androecial tubes. Actual number of androecial ovules per flower was 0-6.

Abbreviations for Figs. 1 - 63. A = androecial primordia (prior to becoming stamen primordia), AF = floral apex, AN = anther, AP = androecial appendage, AT = androecial tube, B = bracteole or scar from removal of bracteole, C = scar from removal of calyx, CT = calyx tube, F = androecial filament, FN = funinculus, FU = fused region of compound style, I = ovule inner integument, M = floral meristem, MP = micropyle, N = ovule nucellus, O = ovule outer integument, OV = ovary base, OVU = androecial ovule, P = petal, P! = congenitally connate petals, PN = polar nuclei, PT = petalode, S = stamen primordia, SE = sepal, SN = synergid, ST = style, STG = stigma, and STP = stipule.

Figs. 1 - 6. Early bracteole, sepal organogenesis in tissue culture-derived (TC) plants of Hibiscus acetosella. Scale bars = 50 uM. 1 - 3. Initiation of bracteole primordia and delimitation of floral apex. The bracteole primordia shown are uneven in size, and gaps exist in the bracteole whorl (opposite stipules in 1, 2). This condition was common in mature flowers. 4 - 6. Initiation of sepal primordia. Sepal primordia were observed to be unevenly distributed about the periphery of the floral apex and to be nonuniform in size. Gaps were found frequently in the calyx tube (5, arrow), and the calyx tube was sometimes initiated without a distal sepal lobe (6, under labeled bracteole).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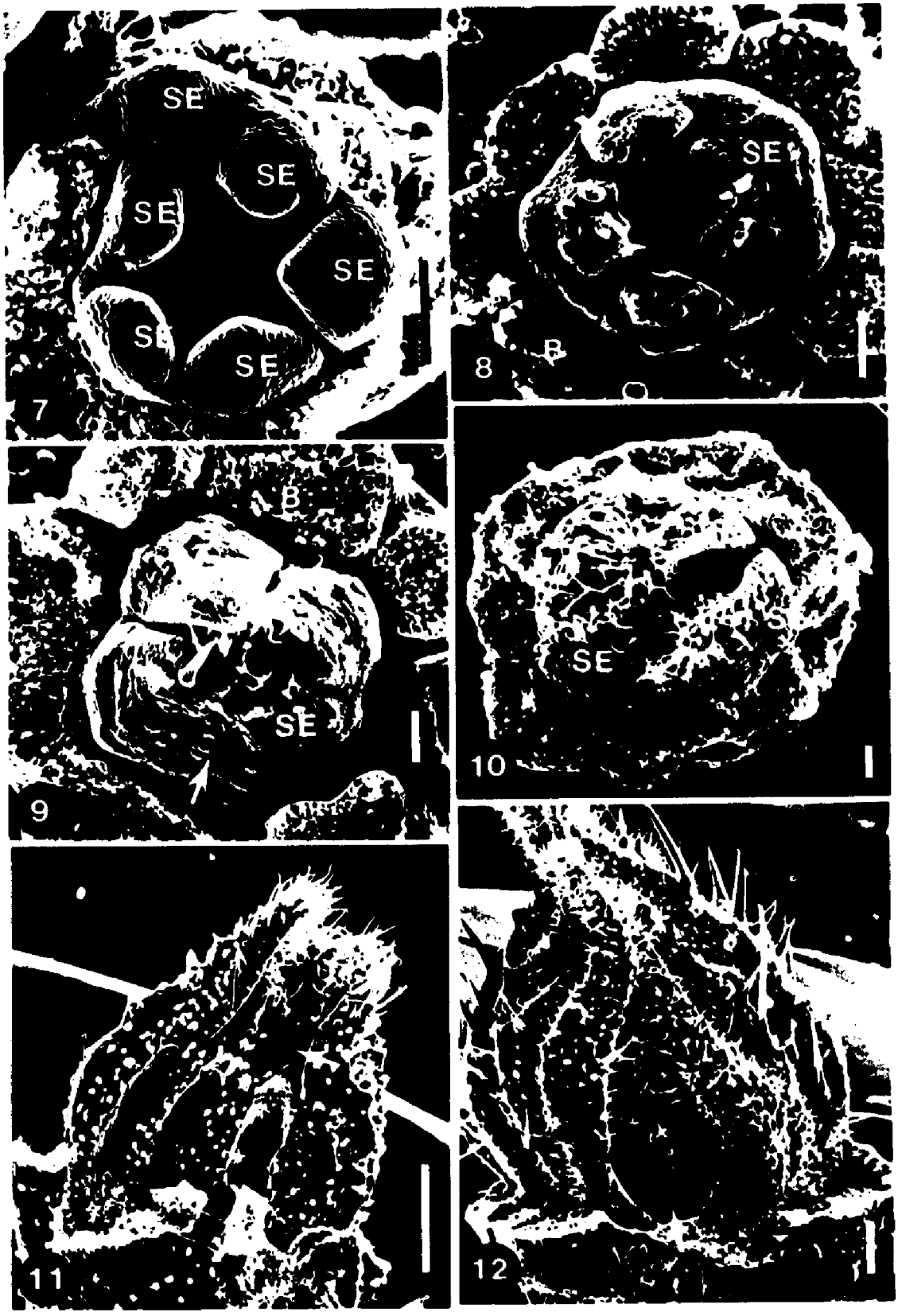
Figs. 7 - 12. Later sepal organogenesis in TC plants of H. acetosella. Bracteoles removed in all figures. Scale bars = 100 uM for 7 - 10, scale bars = 500 uM for 11, 12. 7. Six sepal primordia, usual number is five. 8. Five sepal primordia which underwent unequal lateral and centripetal growth, resulting in uneven lateral appression. 9. Either four primordia, one of which has split into a larger and smaller portion, or five primordia, with one lagging or repressed in development (marked by arrow). 10. The unequal size and overlapping of an undeterminable number of sepals has disrupted the normally valvate sepal aestivation. 11. Sepal lobe absent (arrow), with underlying sepal tube present. 12. Gap in the normally continuous calyx tube (arrow).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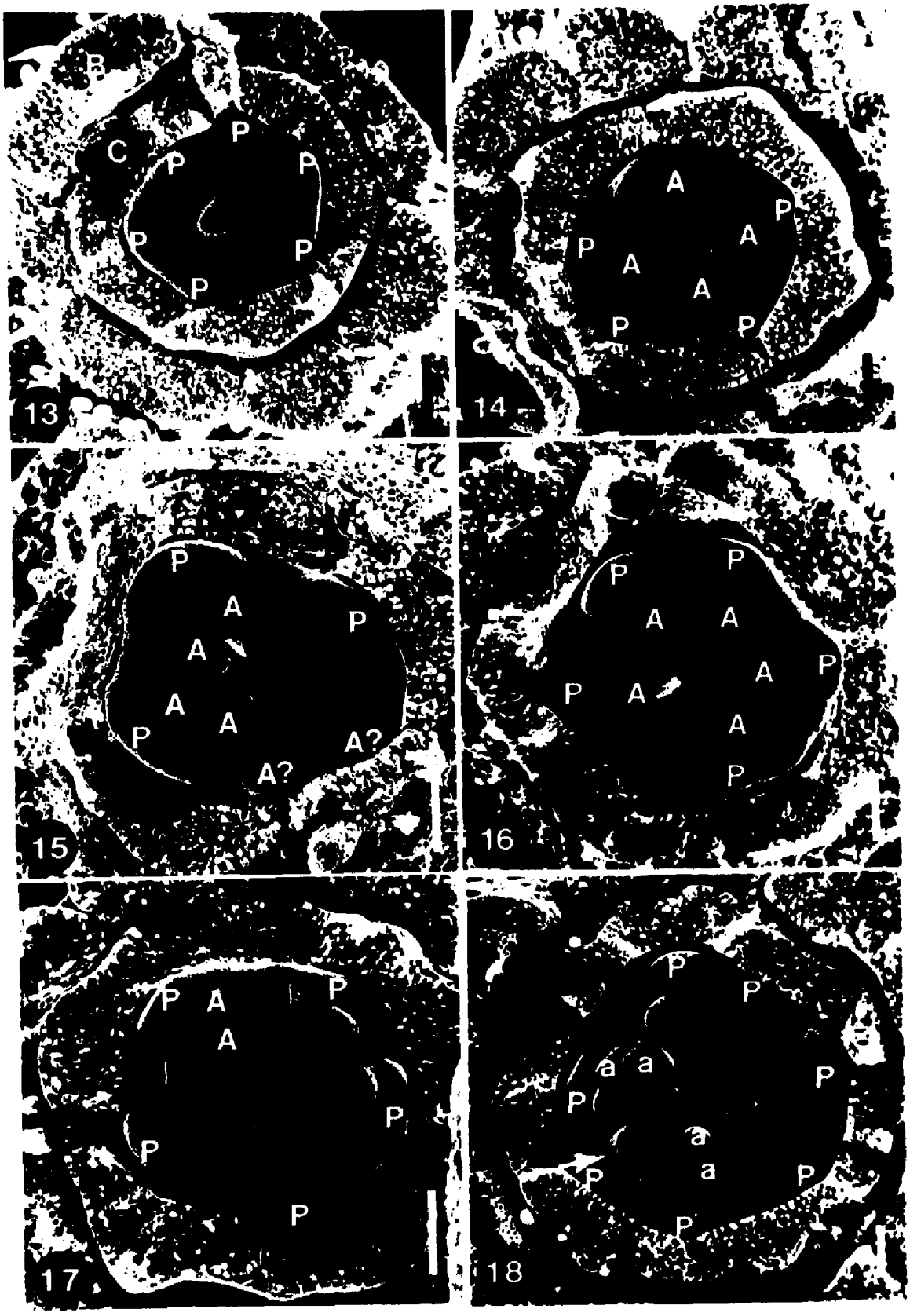
Figs. 13 - 18. Early petal and androecial primordia organogenesis in TC plants of H. acetosella. Bracteoles, sepals removed in all figures. Scale bars = 100 uM. 13. Initiation of six petal primordia, instead of the normal five. 14. Initiation of four petal primordia and of primary androecial primordia. 15. Initiation of three petal primordia, presumptive initiation of secondary androecial primordia (A). Two presumptive androecial primordia (A?) initiated on apex periphery without an adjacent petal primordia. 16. Formation of five petal and five primary androecial primordia as in wild type, but the primordia are of different sizes and are not equally distributed. 17. Five petal primordia as in wild type. Androecial primordia vary in location and number (only two labeled). Divisions in the androecial primordia in this and subsequent figures of androecial development are so anomalous that it is not possible to assign orders (e.g. primary, secondary, etc.) to successive stages of primordia as in the wild type. Fig. 18. Two pairs of petal primordia (P') which resulted from either the connation of two adjacent primordia or from the lateral division of individual primordia. New androecial primordia are initiated centrifugally (arrow). Only four of the radially oriented androecial primordia are labeled.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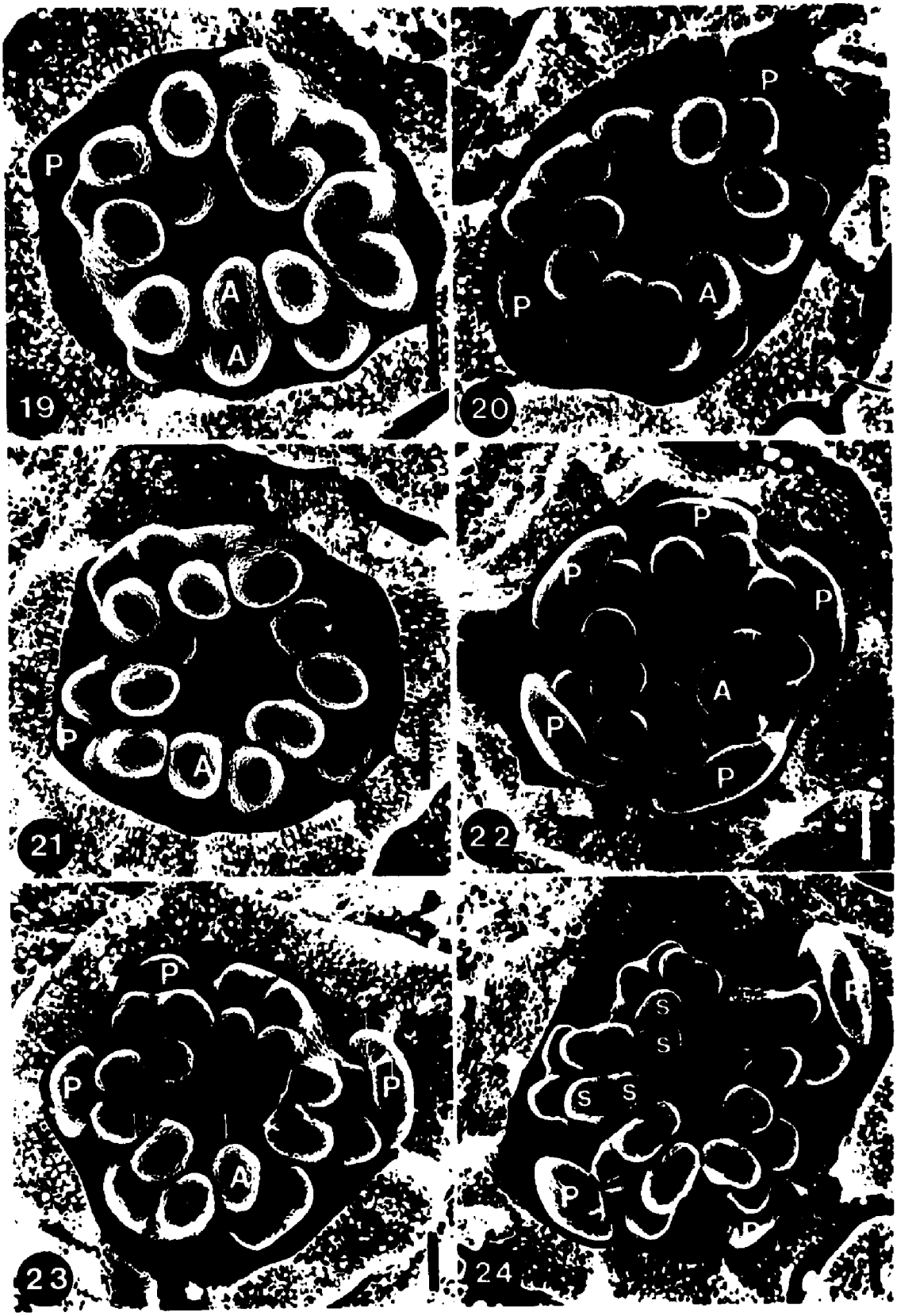
Figs. 19 - 24. Middle developmental stages of petal and androecial primordia organogenesis in TC plants of H. acetosella. Arrows point to centrifugally initiated androecial primordia. Scale bars = 100 uM. 19 - 23. One, two, one, five, and three petal primordia respectively. Androecial primordia irregularly shaped and unevenly distributed, but always centrifugally initiated. 24. Three petals, uneven size and distribution. Several of the androecial primordia are dividing to form pairs of stamen primordia (labeled).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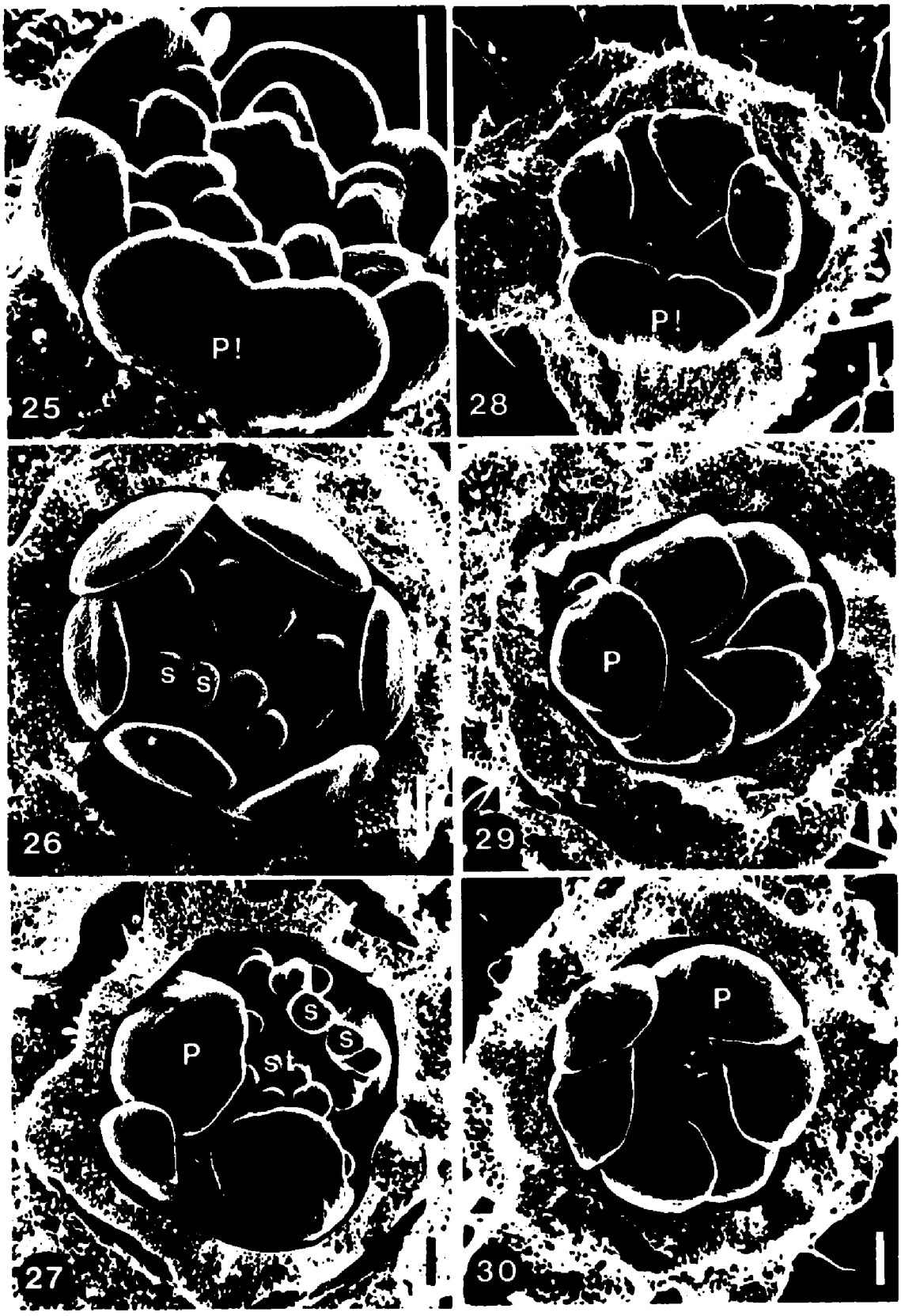
Figs. 25 - 30. Late petal and stamen organogenesis in TC plants of H. acetosella. Scale bars = 150 uM. 25. Lateral view of two congenitally connate petals. 26. Six petal primordia present. Petal aestivation not predictable until the adjacent petal edges expand laterally and overlap (Triangles mark two sites of incipient petal overlap). 27. Three petal primordia, aestivation not predictable, few stamens for such a late stage in development in contrast to wild type. 28. Top view of a flower with more petals than normal, two of which are congenitally connate. Petal aestivation is imbricate, but not convolute, as in wild type. 29, 30. Two flowers each with six petals, but different imbricate patterns of aestivation.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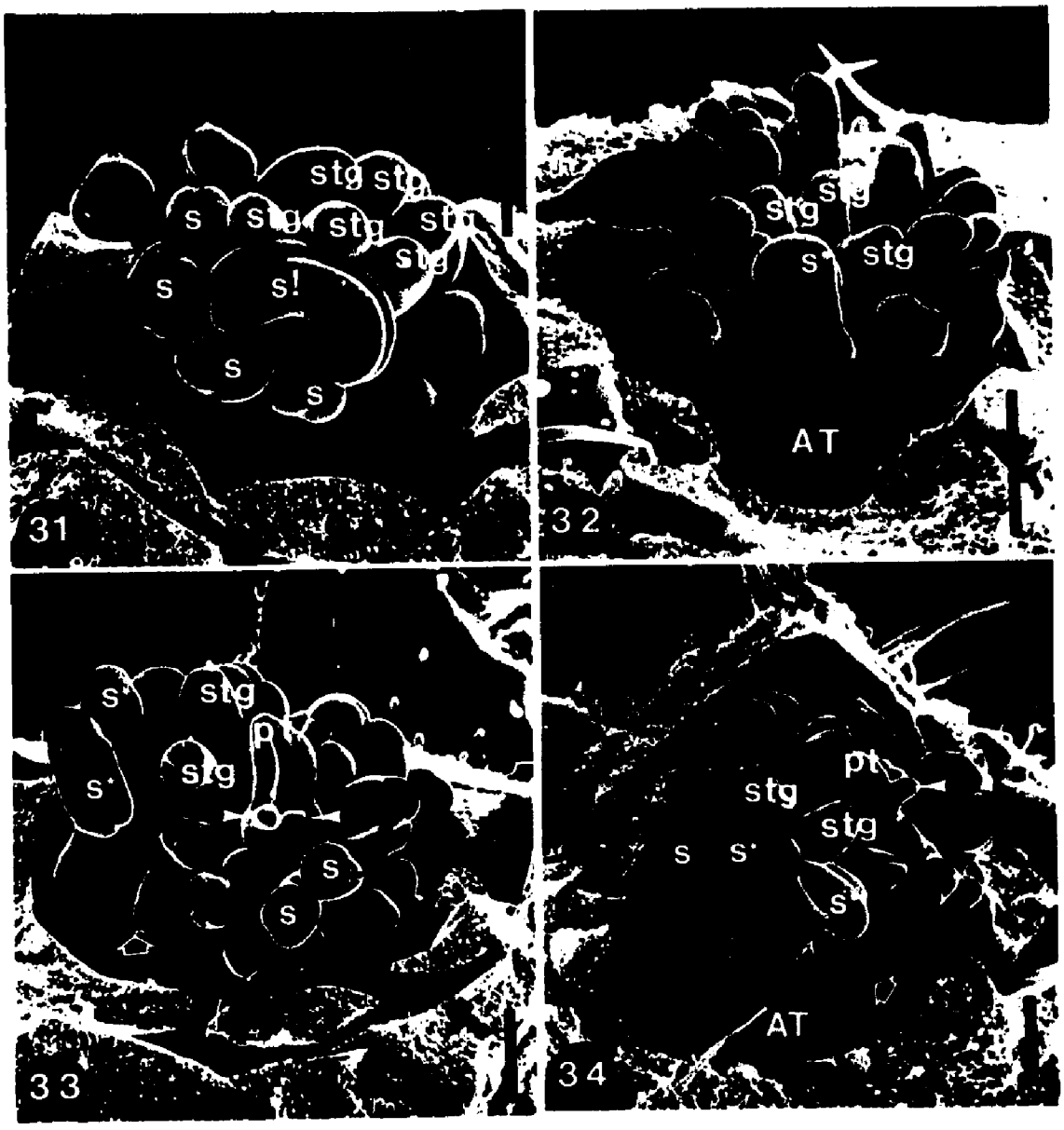
Figs. 31 - 34. Late androecial organogenesis in TC plants of H. acetosella. Bracteoles, calyx, corolla removed. Scale bars = 250 uM. 31. Six style primordia visible, few stamens in contrast with wild type at similar stage of development. One anther (s!) has three sporangia instead of two. 32. Androecium poorly developed for styles already showing. Most of the androecial primordia terminal on the androecial tube, with little or no centrifugal initiation of new primordia. One anther primordium present (S*) on massive filament. 33, 34. Two different views of the same flower, showing break in continuity of androecial tube (arrow outline), with two aberrant anthers (S*) terminal on the portion of noncontiguous androecial tube. The other stamens are few in number and aberrant in morphology. There is a presumptive petalode present, with two lateral anther primordia (arrows).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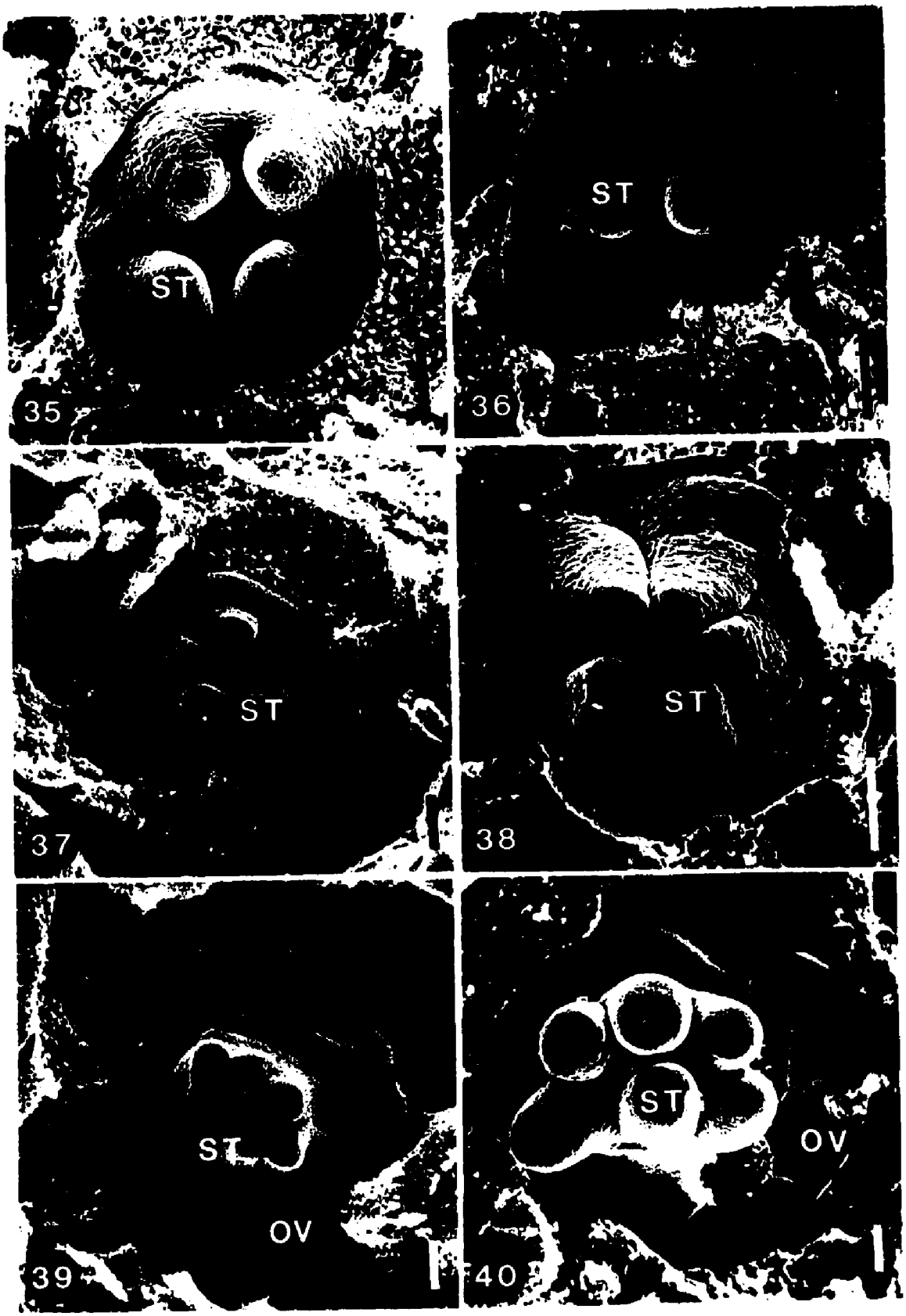
Figs. 35 - 40. Early gynoecial organogenesis in TC plants of H. acetosella. Bracteoles, calyx, corolla, and androecia removed. Scale bars = 100 uM. 35 - 40. The number of carpel primordia varies from four to six, in contrast to five in wild type. Ovary base is congenitally connate in all figures. The abnormal growth of the carpel primordia is evidenced by the unequal appressions between primordia.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Figs. 41 - 44. Middle to late gynoecial organogenesis in TC plants of H. acetosella. Scale bars = 150 uM. 41. Six style primordia of varying size and distribution surrounding another style primordia that may represent carpel initiation in a second, centripetally located whorl. 42. Gynoecium showing incomplete postgenital fusion between six styles. The compound ovary base is connate. 43, 44. Top (43) and lateral (44) view of the same flower. The triangle in 43 marks the side shown in 44. Flower exhibits zygomorphy of the androecium and gynoecium. The androecium primordia in the facing view (44) are centrifugally ordered in two rows opposite a petal, as in wild type, but should have differentiated into anthers by this stage of gynoecial development. The androecial appendages (AP) probably would have remained as sterile filaments. Styles in the wild type postgenitally fuse over almost their entire length, but in this flower are fused only over a short zone (FU), free distally and partially fused or only appressed basally (arrows).


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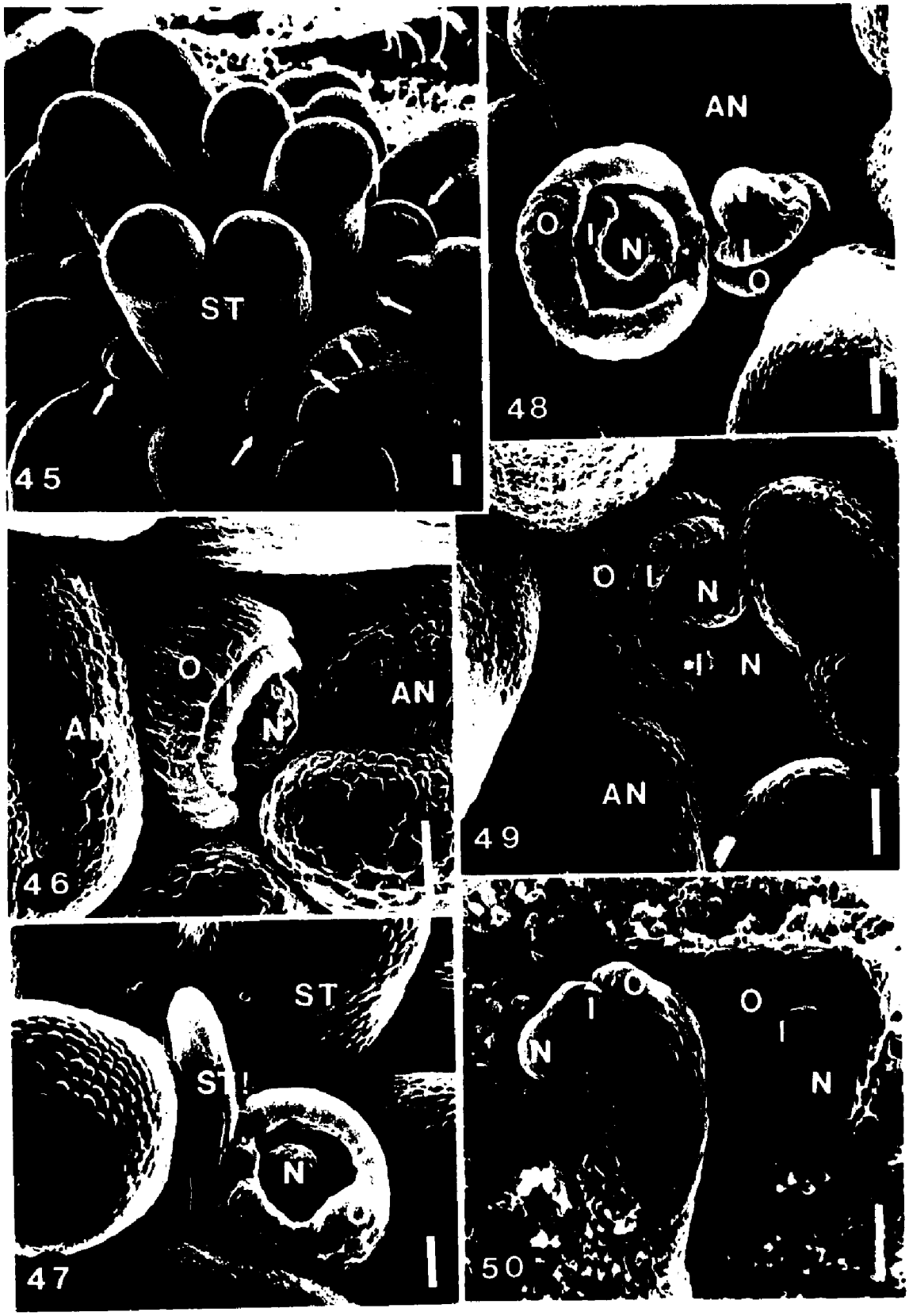
Figs. 45 - 50. Androecial ovule and gynoecial ovule organogenesis in TC plants of H. acetosella. Scale bars = 50 uM. 45. Androecial ovules visible between terminal anthers and stylodes. 46. Androecial ovule from 45, showing nucellus and enveloping outer and inner integuments. 47. Androecial ovule and a presumptive style primordia (ST!) arising from the androecial tube. 48. Two adjacent androecial ovules at different stages of development. The ovule on the left is more advanced in development, as the integuments have almost enveloped the nucellus. 49. An aberrant androecial ovule with one outer integument, two inner integuments, and two nucelli. 50. Zygotic ovules from the same flower as in 45, at a similar stage of development.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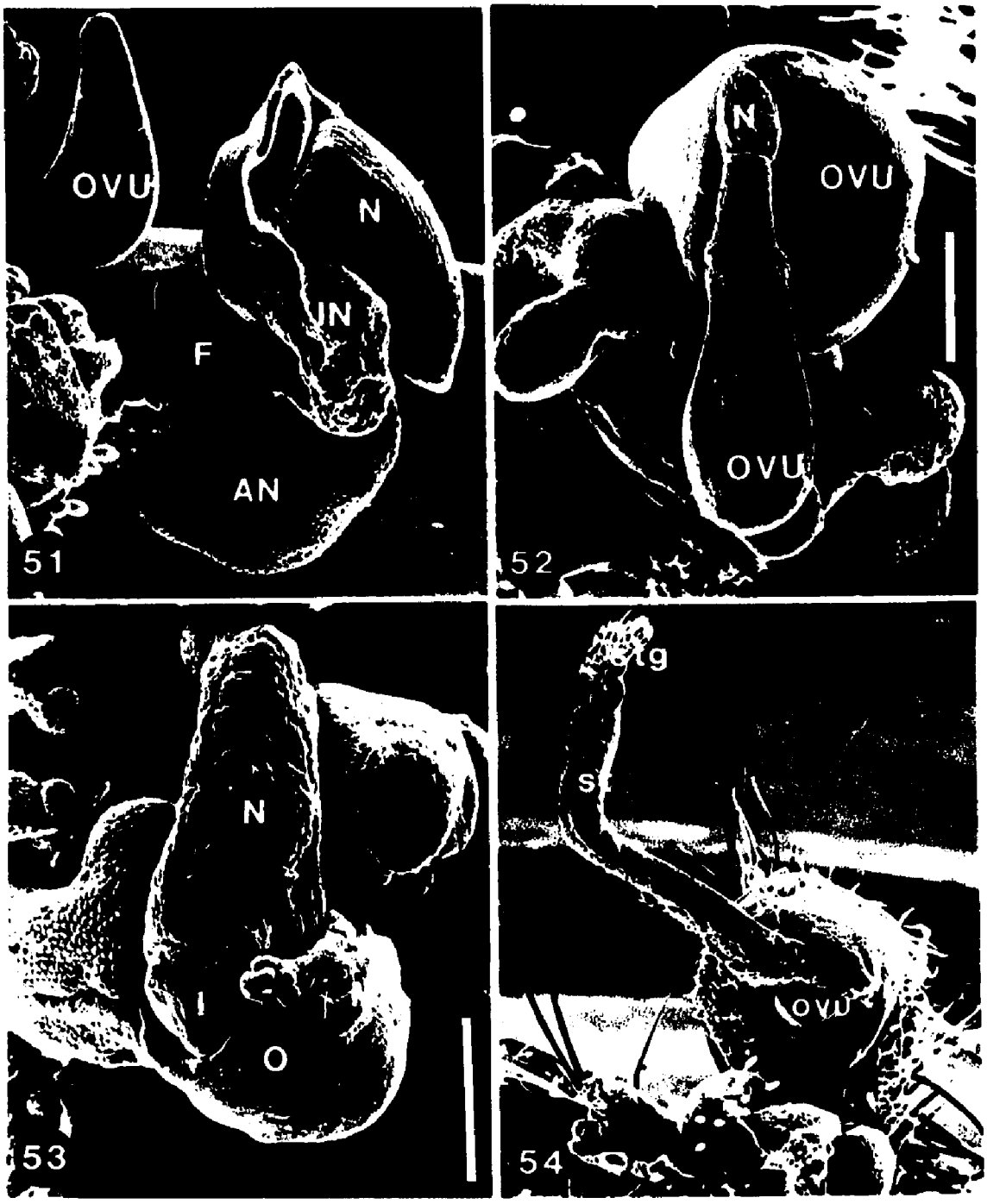
Figs. 51 - 54. Androecial ovule organogenesis in TC plants of H. acetosella. Scale bars = 500 uM. 51. An androecial ovule and an anther terminating a filament of the androecial tube. The two integuments of the ovule are poorly developed, exposing the nucellus. Another ovule with the integuments completely enveloping the nucellus is also present. 52. Two androecial ovules in a terminal position on the androecial tube, showing typical variation in the ovule morphology. The nucellus has grown out of the integuments in one ovule. 53. An aberrant androecial ovule in which the nucellus has grown out of the integuments. The micropylar opening is visible at one end of the ovule. 54. An aberrant carpellate structure on the distal end of an androecial tube, showing the presence of a stigma, style, and one ovule partially enveloped within the carpel.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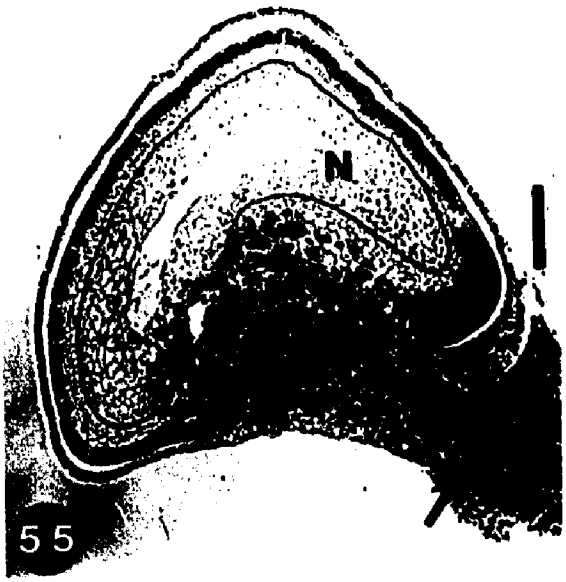
Figs. 55 - 59. Androecial ovule and gynoecial ovule anatomy in TC plants of H. acetosella. Scale bars = 200 uM for 55 - 57, scale bars = 50 uM for 58, 59. 55. Near-median section of a zygotic ovule removed from a flower the day of anthesis. The ovule is bitegmic, crassinucellate, and amphitropous. A single vascular strand (arrow) traverses the funiculus to supply the ovule. 56. Aberrant androecial ovule removed from a flower the day of anthesis with incompletely enveloping outer and inner integuments, and two nucelli. Examination of the other sections of this ovule reveals the left nucellus contains an embryo sac, while the right nucellus does not contain one. 57. Median section through an androecial ovule removed from the flower the day of anthesis, which is similar in appearance to the zygotic ovule in 55. The vascular strand supplying the ovule (not visible in this section) traverses the underlying filament. 58, 59. Portions of the embryo sac from the same androecial ovule, showing the two synergids (58) and the two polar nuclei (59). The egg cell is presumably masked by the synergids.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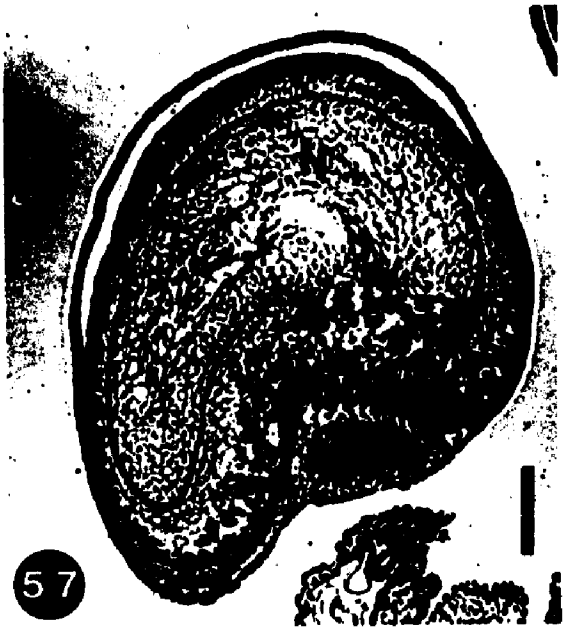
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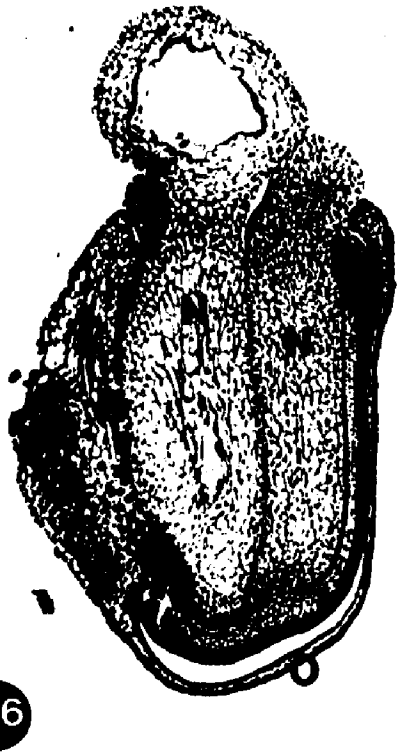
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Figs. 60 - 63. Various floral abnormalities in TC plants of H. acetosella. All flowers removed from plants at anthesis. Scale bars = 1.0 mm. 60. The bracteoles are all fused into a connate structure. The number of teeth indicate that the fused structure may have arisen from seven or eight bracteoles. 61. Typical arrangement of the androecial ovules, distal on the androecial tube, and below the stigmas. 62, 63. Two petalodes arising from the androecial tube (62). A functional anther (arrow, 63) is sessile on the margin of the larger petalode (anther not visible in 62).


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## VITA

The author was born to Robert and Joan Ault on December 22, 1955, in Huntsville, Alabama. His primary and secondary education was acquired in Dayton, Ohio, and Warren, Michigan.

After graduation from Paul K. Cousino High School in Warren, Michigan, he entered the University of Michigan and was granted a Bachelor of Science in Botany in 1978.

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The author married Carolla Rose Jeffery in 1984.

## DOCTORAL EXAMINATION AND DISSERTATION REPORT

**Candidate:** James Robert Ault

**Major Field:** Plant Health

Title of Dissertation: Somaclonal Variation in Hibiscus acetosella Welw. ex Hiern: Altered Fertility and Floral Ontogeny

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

January 29, 1987